Mechanisms for the Selective Actions of Vitamin D Analogs

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Vitamin D was first identified as an essential factor for normal mineral metabolism and skeletal development. These actions are now known to be mediated by metabolites of vitamin D, primarily 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] but perhaps 24,25-dihydroxyvitamin D_3 as well. 1,25(OH)$_2$D$_3$ acts as a steroid hormone and binds to a well-characterized intracellular receptor that is localized in the nucleus and regulates gene transcription by binding to specific motifs within the target genes (see Chapters 11–22). There is also *in vitro* evidence that 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ can interact with cell surface receptors and rapidly stimulate signaling from the cell membrane [1] (see Chapter 23). At present, the potential roles for these nongenomic actions and their relevance *in vivo* are less well understood.

Research from many laboratories has demonstrated that the actions of the vitamin D system extend beyond a role restricted to bone and mineral metabolism. Many of these activities suggested potential therapeutic applications for 1,25(OH)$_2$D$_3$. The native vitamin D hormone is currently in use for the treament of secondary hyperparathyroidism in renal failure patients [2], psoriasis [3], and X-linked hypophosphatemic rickets [4]. In addition, the ability of 1,25(OH)$_2$D$_3$ to block proliferation of many cell types, including neoplastic cells, *in vitro* has indicated the potential of this compound for treating various types of cancer [5] (see Chapters 88–97). However, a major limitation to 1,25(OH)$_2$D$_3$ therapy is its potent calcemic and phosphatemic activities. Studies to date suggest that the doses of 1,25(OH)$_2$D$_3$ necessary to block cell proliferation *in vivo* may also produce profound hypercalcemia and hyperphosphatemia.

I. IDENTIFICATION OF SELECTIVE VITAMIN D ANALOGS

A major breakthrough in vitamin D therapeutics has been the development of vitamin D analogs that retain many of the potential clinically useful activities of 1,25(OH)$_2$D$_3$ for the newly discovered indications, but have much lower calcemic and phosphatemic activities *in vivo* [6,7]. The promising analogs identified initially displayed high differentiating and antiproliferative activities *in vitro*, but were found to have low calcemic activity *in vivo* [8–12]. One explanation for these results could have been that the analogs were relatively less active than 1,25(OH)$_2$D$_3$ *in vivo* due to reduced bioavailability. Although this may be the case for some analogs, several "noncalcemic" analogs [actually, the analogs are all calcemic but to a lesser degree than 1,25(OH)$_2$D$_3$] have been found to retain some of the activities of 1,25(OH)$_2$D$_3$ in animal models. Selective actions of several analogs have now been demonstrated *in vivo*. These analogs are discussed in detail in Chapters 79–87, and their uses for specific indications are discussed in Chapters 88–104.

Secondary hyperparathyroidism in chronic renal failure has been treated with 1,25(OH)$_2$D$_3$ or 1α(OH)D$_3$ for many years, but the potent calcemic activities of these compounds often produce hypercalcemia. Several new analogs have been developed that retain the PTH suppressive effect of 1,25(OH)$_2$D$_3$ but with lower calcemic activity. Four analogs are currently available to patients: 1,25(OH)$_2$-19-nor-D$_2$ (Abbott Laboratories) and 1α(OH)D$_2$ (Bone Care International) in the United States and 22-oxa-1,25(OH)$_2$D$_3$ (OCT; Chugai Pharmaceuticals) and 1,25(OH)$_2$-26,27-F$_6$-D$_3$ in

Japan [13] (see Chapters 75, 85, 86, and 103). Studies in animals have revealed that OCT effectively suppressed parathyroid hormone (PTH) levels in hyperparathyroid uremic rats at doses that had minimal effects on serum calcium and phosphate levels [14–16]. Data from the study of Hirata *et al.* [16] demonstrating the selectivity of OCT are illustrated in Fig. 1. Similarly, $1,25(OH)_2$-19-nor-D_2 was found to be about three times less active than $1,25(OH)_2D_3$ in suppressing PTH in patients, but ten times less calcemic and phosphatemic [17–21]. Renal failure patients receiving $1,25(OH)_2$-19-nor-D_2 have been found to have lower rates of mortality than those receiving $1,25(OH)_2D_3$ [22]; the mechanism is unclear but may involve the lower calcemic and phosphatemic activities of the hormone. Selective effects *in vivo* of $1\alpha(OH)D_2$ and $1,25(OH)_2$-26,27-F_6-D_3 on the parathyroid glands have not been reported. Other analogs shown to display parathyroid selectivity in animal models include 1,25-dihydroxy-dihydrotachysterol [23] and the 20-epi analogs CB1093, EB1213,

and GS1725 [24]. Clearly, it is possible to develop analogs with selectively for suppression of PTH.

Analogs are also available for treatment of psoriasis. Calcipotriol, the first of the "noncalcemic" analogs to be approved for clinical use, is effective in treating psoriatic lesions by topical application, and has minimal calcemic activity, even when administered systemically [10,25]. $1,24(OH)_2D_3$ and OCT are also available in Japan for psoriasis [26,27]. Although toxicity of topically applied vitamin D compounds is less compared to systemic administration, excessive application can cause hypercalcemia and therefore the lower calcemic activities of the new analogs provide a safer means to treat psoriasis.

The greatest activity for vitamin D analog therapy is in the treatment of various types of cancer, as discussed in greater detail in Section IX. 22-Oxa-l,$25(OH)_2D_3$ and the analog EB1089 (Leo Pharmaceuticals) were shown to suppress PTH-related peptide in cancer cells *in vivo* [28,29], suggesting their use in the treatment of
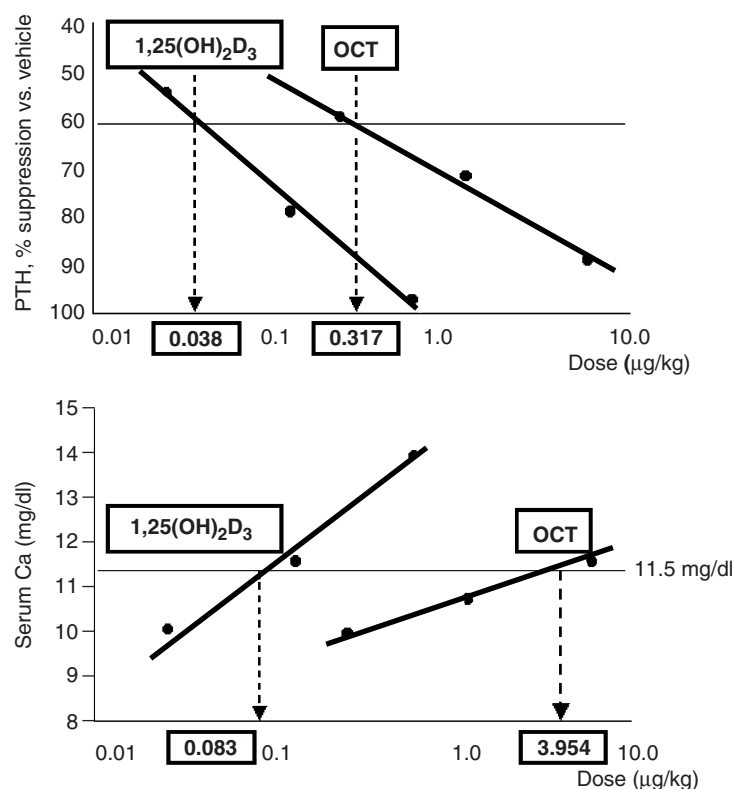

FIGURE 1 Selectivity of OCT for suppression of PTH in uremic rats. Uremic rats were treated with OCT or $1,25(OH)_2D_3$ at the specified IV doses every other day for 2 weeks, and PTH and calcium were determined 24 hours after the final injection. The doses of each compound that produced a 40% suppression of PTH or an increase in calcium to 11.5 mg/dl are shown. OCT was 8.3 times less potent in suppressing PTH, but 47.6 times less calcemic, indicating that the therapeutic window is approximately 5.7 times wider for OCT than for $1,25(OH)_2D_3$. Adapted from Hirata *et al.* [16].

hypercalcemia of malignancy. 22-Oxa-l,25(OH)$_2$D$_3$ [30,31], calcipotriol [32], and EB1089 [33] (Leo Pharmaceuticals), l,25-(OH)$_2$-16-ene-23-yne-26,27-F$_6$-vitamin D$_3$ [34] (Hoffmann-LaRoche), and 1α(OH)D$_5$ [35] have been shown to inhibit the *in vivo* proliferation of breast cancer cells. The analogs KH1060, EB1089, and Ro 26-9114 were shown to control prostate cancer cell (LNCaP) growth in nude mice more effectively than 1,25(OH)$_2$D$_3$, inducing tumor necrosis and calcification, but with no hypercalcemia [36]. The analog l,25-(OH)$_2$-16-ene-23-yne-26,27-F$_6$-vitamin D$_3$ may be effective in control of androgen-induced carcinoma of the prostate and seminal vesicles [37,38]. 22-Oxa-1,25(OH)$_2$D$_3$ [39] and l,25-(OH)$_2$-16-ene-23-yne-26,27-F$_6$-vitamin D$_3$ [40], and 1,25(OH)$_2$-16-ene-19-nor-24-oxo-D$_3$ [41] can inhibit growth of experimentally induced tumors of the small and large intestine. Another derivative, l,25-(OH)$_2$-16-ene-23-yne-vitamin D$_3$ (Hoffmann-LaRoche), can prolong the survival of mice injected with leukemia cells [42]. EB1089 was found to give a greater reduction in tumor size than 1,25(OH)$_2$D$_3$ in a mouse model of head and neck squamous cell carcinoma without producing hypercalcemia [43]. Many other analogs with low calcemic activity that have been shown to inhibit proliferation of cancer cells *in vitro* have not yet been demonstrated to exert selectivity *in vivo*.

The immunomodulatory actions of vitamin D compounds have suggested therapeutic applications for autoimmune disorders and transplantation. Modulation of the immune system with little hypercalcemia has now been demonstrated for several analogs. Abe *et al.* found that OCT was 50 times more potent than 1,25(OH)$_2$D$_3$ in augmenting a primary immune response in mice, but was 100 times less calcemic [44]. Lemire *et al.* demonstrated that 1,25(OH)$_2$-16-ene-24-oxo-D$_3$ was more potent than 1,25(OH)$_2$D$_3$ or 1,25(OH)$_2$-16-ene-D$_3$ in suppressing experimental autoimmune encephalomyelitis, but, unlike the other two compounds, did not increase serum calcium [45]. KH1060 was shown by Mathieu *et al.* to reduce the incidence of diabetes in NOD mice at doses that did not increase serum or urinary calcium [46]. Most recently, Zugel *et al.* showed that the vitamin D analog ZK191784 effectively inhibited contact hypersensitivity in mice [47]. Although it was approximately 100 times less active than 1,25(OH)$_2$D$_3$, its effect on urinary calcium was more than 3000 times lower. These studies clearly illustrate the selectivity of the analogs on the immune system.

Osteoporosis is another therapeutic target for vitamin D analogs. 1,25(OH)$_2$D$_3$ or its synthetic analog 1α(OH)D$_3$ have been used with some success, but analogs are being developed that are more effective in increasing bone mineral. One such analog with clinical potential is 1,25(OH)$_2$-2-(3-hydroxypropoxy)-D$_3$ (ED-71) from Chugai Pharmaceuticals. This analog appears to be much more effective than 1,25(OH)$_2$D$_3$ and 1α(OH)D$_3$ in stimulating bone mineralization in ovariectomized rats [48,49] and in corticosteroid-treated rats [50]. ED-71 is currently in clinical trials in Japan. A very promising new analog for osteoporosis is Ro-26-9228. This highly modified compound (1F,25(OH)-20-epi-23-ene-25,26-dimethyl-D$_3$) is bone protective *in vivo*, and has been shown to be very active in bone but not in the duodenum [51]. Similar selectivity was noted for 2-methylene-19-nor-(20S)-1,25(OH)$_2$D$_3$ (2MD), which showed greater relative activity in bone than in the intestine. In OVX rats, 2MD increased bone mass whereas 1,25(OH)$_2$D$_3$ only prevented loss of bone mineral [52]. The mechanism responsible for the cell/tissue specificity of these analogs is under investigation.

In general, the low calcemic activity of these analogs permitted the use of doses sufficiently high to obtain the desired effect. Quantification of the degree of selectivity (i.e., potency of the desired effect versus calcemic activity) is difficult to determine in many cases because of lack of data for the relative dose responses for 1,25(OH)$_2$D$_3$ and the analog. In some studies, such data cannot be gathered due to the hypercalcemic toxicity of 1,25(OH)$_2$D$_3$ at the doses required for the desired activity.

The novel aspect of these analogs is their differential actions, compared to 1,25(OH)$_2$D$_3$, *in vivo*. In fact, as these analogs have relatively high affinity for the vitamin D receptor (VDR), usually within one order of magnitude, it is not unexpected that they are able to mimic many of the actions of 1,25(OH)$_2$D$_3$ *in vivo*. Their unique feature is the ability to efficiently support some but not all l,25(OH)$_2$D$_3$-associated activities. Most commonly, the analogs display decreased potency in enhancing intestinal calcium absorption and/or bone mobilization. In some cases, the analogs have relatively high calcemic effects, but tend to produce even higher activities in other specific cells or tissues. The selectivity is not always cell- or tissue-specific, but can be gene- or process-specific within the same tissue. For example, several analogs [l,25(OH)$_2$-22-ene-24-dihomo-D$_3$, 1,25(OH)$_2$22-ene-24-trihomo-D$_3$, and 1,25,28(OH)$_3$D$_2$] have been shown to induce the vitamin D–dependent calcium-binding protein in the intestine without increasing intestinal calcium transport [53,54]. Similarly, 20-epi-l,25(OH)$_2$D$_3$ was found to be 1000 times more potent than 1,25(OH)$_2$D$_3$ in inducing cell differentiation in a human leukemia cell line (HL-60), but it was equipotent in activating transcription of a vitamin D–responsive reporter construct in the same cells [55]. The remainder of this chapter

discusses potential mechanisms for the selective actions of vitamin D analogs, presenting examples when available.

II. THE *IN VIVO* SELECTIVITY OF VITAMIN D ANALOGS IS DETERMINED BY MULTIPLE PROTEIN INTERACTIONS

The potential mechanisms through which selectivity could be achieved are summarized schematically in Fig. 2. These include altered systemic transport via DBP (1) or lipoproteins (2) that could influence cellular uptake (3); interaction with intracellular vitamin D binding proteins that target the compounds for metabolism or to the VDR; metabolism to active (4) or inactive (5) compounds; altered binding to the plasma membrane receptor (6); induction by the analog or its metabolites of an altered conformational change in the VDR (7) that could influence heterodimerization with RXR (8), binding to DNA (9) and subsequent recruitment of other components to the transcriptional initiation complex (10). The factors that contribute to selectivity of a few vitamin D analogs have been identified, and in many cases multiple factors may be involved.

As a general concept, the actions of vitamin D compounds are determined by their interactions with five classes of proteins: the nuclear vitamin D receptor, transport proteins (primarily the serum vitamin D binding protein), metabolic enzymes (the vitamin D-24-hydroxylase and the 3-epimerase), cell surface membrane receptors, and the newly discovered intracellular vitamin D–binding proteins. The following sections discuss the structural modifications in vitamin D analogs that can affect these interactions and, ultimately, the biological profile of the compounds.

A. Vitamin D Receptor (VDR)

Most of the biological activities of vitamin D compounds are mediated by a nuclear receptor that binds $1,25(OH)_2D_3$ with high affinity and specificity. The observation that some vitamin D analogs exerted selective actions *in vivo* (i.e., therapeutic activity similar to or higher than that of $1,25(OH)_2D_3$, but with lower calcemic activity) led to early speculation that there could be multiple forms of the VDR that may recognize some but not all vitamin D compounds. It was proposed that the intestine and bone may express a form of the
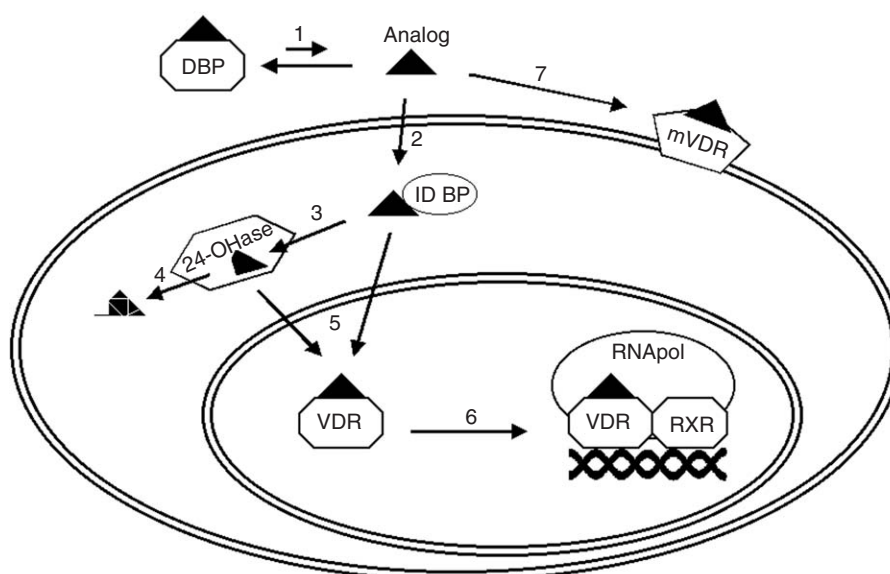

FIGURE 2　Potential sites of differential actions of $1,25(OH)_2D_3$ and its analogs. Possible steps in the vitamin D activation pathways at which differences in vitamin D analog action could lead to selective activities *in vivo* are shown. The steps diagrammed include: (1) interactions with DBP or other serum proteins including lipoproteins, (2) cellular uptake and interaction with intracellular binding proteins, (3) intracelluar metabolism to active intermediary metabolites or (4) to inactive end-products, or (5) nuclear uptake and VDR binding (6) formation of the VDR-RXR complex, binding to the activated complex to DNA and formation of the preinitiation complex RNA polymerase II (RNApol) and (7) activation of the nongenomic pathway through a putative membrane vitamin D receptor (mVDR).

VDR that discriminates against the noncalcemic analogs. Presently there is no evidence that multiple forms of the VDR are responsible for the altered actions of certain analogs. However, the report by Crofts *et al.* [56] revealed that the VDR gene is driven by multiple promoters that incorporate different 5′ exons into the VDR mRNA, which could result in VDR proteins with different N-termini. The expression of the bulk of these transcripts was very low, however, and varied with cell type and tissue. At present, it is unclear whether cell-specific expression of these forms of the VDR account for the differential effects of vitamin D analogs observed *in vivo*.

Vitamin D ligands induce a conformational change in the receptor upon binding. This is discussed in detail in several other chapters in this volume. The altered structures of vitamin D analogs likely contact the VDR differently than $1,25(OH)_2D_3$, perhaps leading to subtle changes in the active VDR conformation that could potentially produce cell or gene-specific selectivity [7].

Steroid hormone receptors are complex molecules that interact with small molecules (ligands), many other proteins (kinases, motor proteins, other transcription factors, and components of the transcriptional initiation complex), and very specific DNA sequences. Not surprisingly, VDR and the members of this receptor superfamily contain multiple functional domains. The locations and functions of these domains in the VDR molecule are discussed in detail elsewhere in this volume (see Section II). The following sections present potential mechanisms by which differential interactions of vitamin D analogs with the VDR could lead to selective actions at the cellular or gene level.

1. LIGAND BINDING

Therapeutically useful vitamin D analogs must bind well to the VDR, at least in the clinically relevant target tissue(s) or cell(s). The key structural portion of the vitamin D compound for VDR binding is the A-ring containing the 1α-hydroxyl group. Until recently, all evidence indicated that the 1α-hydroxyl group was essential for VDR binding and activation. Modification of other parts of the molecule, however, appears capable of restoring biological activity in the absence of the 1α-hydroxyl group. Gardner and co-workers [57] reported that hexafluorination of carbons 26 and 27 can partially restore the HL-60 differentiating activity of several 1-desoxy analogs. The basis for these findings is unclear, but 1-hydroxylation of the analogs by these cells cannot be excluded. However, this explanation cannot account for the observation by Peleg *et al.* [58] that epimerization of carbon 20 can restore the transcriptional activity of 1β-hydroxymethyl-$1,25(OH)_2D_3$.

Thus, other modifications appear to be capable of compensating for the lack of a functional 1α-hydroxyl group. Other portions of the molecule, notably the side chain, can be greatly modified with minimal effect on VDR binding. In fact, a recently reported analog lacking the entire side chain ((20S)-1α-hydroxy-2-methylene-19-nor-bishomopregnacalciferol) is able to effectively suppress PTH *in vivo* with virtually no calcemic activity [59]. Other analogs that lack the C ring have been shown to retain high VDR affinity and activity [60]. However, as discussed below, the strength of the interaction between the VDR and its ligand is not the only determinant of VDR-mediated activation.

The affinity of vitamin D compounds for the VDR is often a good predictor of their activity, but many exceptions can be found. Notably, several analogs with the 20-epi configuration have much higher activity than expected based on their equilibrium binding affinity (Kd). It is important to remember that the Kd is simply a ratio of the rate constants for dissociation and association (Kd = k_{dissoc}/k_{assoc}). Further kinetic analysis revealed that several (and perhaps all) 20-epi analogs have low rates of dissociation, implying that the rate of association is also low. The major effect of a slow dissociation is greater stabilization of the VDR; the impact of this is discussed below in more detail. This example illustrates the potential limitations of estimating the functional potential of an analog on the basis of its equilibrium binding.

VDR affinity may also differ between cell types *in vivo*. Koike *et al.* [61] performed autoradiography on tissues from rats injected with varying doses of [^{3}H]OCT. They observed saturation of the tritium localization to the parathyroid glands at lower concentrations than in other tissues. The mechanism for this lower apparent Kd for the VDR in the parathyroid gland is unclear. The VDR in the parathyroid glands is identical to that in other tissues, and therefore the higher apparent affinity is likely due to other factors such as the intracellular binding proteins that appear to play a role in the delivery of ligands to the VDR (see below).

The ligand-binding domain (LBD) is located in the carboxy-terminal half of the VDR. At the C-terminal end of the LBD is the activation function-2 domain (AF-2). This terminal α-helix undergoes a dramatic conformational shift upon binding of the ligand, a crucial step in receptor activation. As discussed below and elsewhere in this volume (see Section II), several vitamin D analogs have been shown to induce a different VDR conformation than $1,25(OH)_2D_3$. As a result, each of the following ligand-dependent processes may be differentially affected by vitamin D analogs.

2. TRANSLOCATION OF THE VDR TO THE NUCLEUS

Ligand binding leads to nuclear translocation of the VDR from the cytoplasm to the nucleus, a process discussed in detail in Chapter 22. Two potential nuclear translocation motifs have been identified in the VDR molecule. One is a bipartate motif consisting of basic residues at each end of the sequence spanning amino acids 79 to 105 in the human VDR. The other is a basic sequence of seven amino acids [49–55] that is unique to the VDR. Point mutations in these sequences impair VDR translocation and activity. The VDR is rapidly shuttled to the nucleus along microtubules in response to $1,25(OH)_2D_3$ in cultured fibroblasts and monocytes [62,63]. This was confirmed by following, in real time, the ligand-dependent translocation of a fusion protein of VDR with green fluorescent protein (GFP) [64]. Structural analysis revealed the requirement of the AF-2 domain of the VDR. It is likely that the VDR interacts with a motor protein that directs it to the nucleus. However, it is unclear whether this interaction is stimulated by a ligand-induced conformational change in the VDR or by activation of the process by ligand-dependent signaling via a membrane receptor (e.g. stimulation of kinases that activate the motor proteins). By either mechanism, vitamin D analogs could differentially affect the rate of nuclear uptake of the VDR and, therefore, its ability to control gene transcription.

3. VDR PHOSPHORYLATION

The VDR becomes hyperphosphorylated in response to the ligand [65] (see Chapter 13), but the role of phosphorylation on VDR activity *in vivo* is not fully understood. The major ligand-dependent phosphorylation sites have been identified as Ser51 and Ser208, although other potential phosphorylation sites may be present. As for stimulating nuclear translocation, it is unclear whether the ligand induces VDR phosphorylation by changing the conformation of the receptor to allow access to protein kinases or by stimulating specific kinase cascades through activation of the membrane receptor. The effect of phosphorylation on VDR activity appears to vary with the site modified. *In vitro* studies have shown that phosphorylation of Ser51 by protein kinase C (PKC) [66] leads to a decrease in VDR activity [67,68]. $1,25(OH)_2D_3$ also has been shown to rapidly activate PKC through interaction of the membrane receptor, suggesting that activation of PKC may operate to attenuate the genomic activity of hormone. The VDR also contains a consensus site for protein kinase A [69], but the role of phosphorylation of this site is unclear. Initial studies report enhancement [70,71] and inhibition [69] of transcriptional activity. In support of the latter observation, co-expression of the catalytic subunit of PKA with the VDR in HeLa and Saos-2

cells inhibited transactivation by $1,25(OH)_2D_3$, but mutation of the PKA site on the VDR did not affect the inhibitory effect of PKA, suggesting that phosphorylation of other proteins, not the VDR, is responsible [72]. Phosphorylation of Ser208 by casein kinase-II has been shown to increase the transcriptional activity of the VDR [73]. The overall role of serine phosphorylation of the VDR appears to be stimulatory. Okadaic acid, an inhibitor of the serine/threonine protein phosphatase-1, enhances VDR-mediated transactivation [74,75]. Tyrosine phosphorylation of the VDR has also been reported. In skeletal muscle cells, $1,25(OH)_2D_3$ has been shown to rapidly activate Src kinase leading to Src interaction with and phosphorylation of the VDR [76]. The effect of tyrosine phosphorylation on VDR activity is not clear, but it may allow interaction of the receptor with proteins involved in rapid signaling. Thus, phosphorylation of the VDR may regulate its activity. At present there is no evidence that vitamin D analogs differentially affect VDR phosphorylation. However, given that $1,25(OH)_2D_3$ may alter the activity of the kinases responsible for VDR phosphorylation by stimulation of nongenomic pathways mediated by membrane receptors with clearly different ligand specificities, it seems likely that analogs would vary in their abilities to alter the phosphorylation state, and therefore activity, of the VDR independently of their actual affinity for the VDR.

4. HETERODIMERIZATION OF THE VDR WITH RXR

Like the other members of the steroid/thyroid receptor superfamily, the liganded VDR binds to DNA motifs (vitamin D response elements or VDREs) not as a monomer, but as part of a multiprotein complex. Binding to most known VDREs is greatly enhanced by the ligand-dependent interaction of the VDR with another nuclear transcription factor, the retinoid X receptor (RXR), but some VDREs may recognize VDR complex lacking RXR [77]. It has been postulated that VDR can also form homodimers as well, but this has been demonstrated only *in vitro* with much higher, and likely nonphysiological concentrations of VDR. The portion of the VDR that interacts with RXR involves the ligand-binding domain, and the interaction could be greatly affected by the type of conformational change induced by $1,25(OH)_2D_3$ or its analogs or by steric hindrance by the ligand itself. Liu *et al.* reported that 20-epi-$1,25(OH)_2D_3$ is more potent than $1,25(OH)_2D_3$ in promoting VDR-RXR dimerization, in keeping with the higher activity of this analog [78]. Enhanced promotion by 20-epi analogs of VDR homodimerization has also been reported [79]. While the role of VDR homodimers is uncertain, this provides a potential mechanism for

analog-enhanced activation of genes with VDREs that respond to the homodimer.

5. DNA BINDING

The ligand-activated VDR interacts with specific DNA motifs in the promoters of target genes termed vitamin D response elements (VDREs). Promoter analysis of genes regulated transcriptionally by $1,25(OH)_2D_3$ has identified many VDREs with similar but distinct structure [80–82] (see Section II). The most common VDRE type, designated DR3, contains two direct repeats of 6 nucleotide bases separated by a 3-nucleotide spacer. The sequence of the hexameric repeats, or half-sites, varies considerably, but a general consensus sequence of AGGTCA has been established. In general, the RXR binds to the upstream half-site and the VDR to the downstream half-site. Another type of VDRE, the IP9, consists of two inverted palindromic sequences separated by 9 base pairs [83].

Vitamin D ligands have been shown to influence both the strength and specificity of the interaction of the VDR/RXR heterodimer with DNA. There is now considerable evidence that $1,25(OH)_2D_3$ analogs with the 20-epi configuration induce a stronger interaction between the VDR and RXR *in vitro*, leading to a potentially more stable association of the heterodimer with VDREs. Studies by Peleg and co-workers [84] demonstrated that several 20-epi vitamin D analogs have higher activity than predicted from their VDR affinities. Using electrophoretic mobility shift assays (EMSA), which measure binding of proteins to small DNA molecules, these authors found increased binding of VDR/RXR to the osteocalcin VDRE in nuclear extracts from cells treated with 20-epi analogs than in extracts from cells treated with $1,25(OH)_2D_3$. Furthermore, they provided evidence that the 20-epi analogs produced a conformation in the VDR that was distinct from that induced by $1,25(OH)_2D_3$, using an *in vitro* protease-clipping assay in which recombinant, ^{35}S-labeled VDR bound to various ligands is treated with a protease and the fragments are resolved by electrophoresis. The 20-epi analogs protected the VDR from proteolysis in a way that is different from that of $1,25(OH)_2D_3$, suggesting an altered conformational state. As stated above, the C-terminus of the VDR (AF-2 domain) is essential for binding of other critical proteins and for transcriptional activity. Peleg *et al.* found that elimination of AF-2 domain prevented VDR activation by $1,25(OH)_2D_3$ but not by the 20-epi analogs [85]. Again, these findings point to a distinct conformation induced by the 20-epi compounds that enhances binding of these other proteins, including RXR, in a manner that is independent of the AF-2 domain. These findings were confirmed by Ryhanen *et al.* [86]

who reported that the 20-epi analogs MC1288 and KH1060 are more potent inducers of alkaline phosphatase and osteocalcin in osteoblastic cells than $1,25(OH)_2D_3$ and that the effects were of longer duration. They demonstrated more stable DNA binding of VDR/RXR from nuclear extracts from analog-treated than from $1,25(OH)_2D_3$-treated osteoblasts. Similar findings have been reported for other 20-epi analogs [87]. At least part of this enhanced stability induced by the 20-epi analogs may be attributed to stabilization of the VDR as discussed below.

While these properties of the 20-epi analogs could endow them with greater potency in stimulating VDR-mediated responses, they do not necessarily confer selectivity since the VDR is responsible for both antiproliferative and calcemic activities of vitamin D compounds. For example, despite the extremely potent immunosuppressive potential of KH1060 observed *in vitro*, the analog did not prevent renal allograft rejection or the development of hypercalcemia [88].

The vitamin D ligand affects not only the strength of VDR binding to the VDRE, but also its specificity. Analogs containing a 20-methyl group appear to preferentially activate VDR binding to the IP9 type of VDRE [89]. The analog EB1089 was shown to promote preferential interaction of the VDR with the IP9 type of VDRE, while CB1093 promoted binding preferentially to the DR3 type [90]. This could account for the differential effects of the two analogs on cell growth. While the two compounds are equipotent inhibitors of cell proliferation, CB1093 is 10 times more active in inducing apoptosis. Thus, an analog may determine the activity of the VDR on a gene-specific basis by altering the interaction with specific VDRE motifs.

6. RECRUITMENT OF OTHER COMPONENTS OF THE INITIATION COMPLEX

Once the heterodimer is bound to DNA, it recruits other components of the transcriptional initiation complex (e.g. TFIIB [91]). In addition, steroid receptor coactivators interact with the VDR in a ligand-dependent fashion to further enhance transcriptional activity. The interaction of the VDR with these proteins, and hence its transcriptional activity, could also be influenced by the different conformation states induced by vitamin D analogs.

Several vitamin D analogs have been shown to differ from $1,25(OH)_2D_3$ in their recruitment of these coactivators. The higher potencies of 20-epi analogs in the induction of the cell cycle inhibitor p21 have been attributed, at least in part, to their enhanced abilities to recruit the DRIP coactivator complex to the initiation complex [92]. Again, this could be due to enhanced stabilization of the VDR. On the other hand, the 20-epi

analogs were found to be equipotent to $1,25(OH)_2D_3$ in the recruitment of the coactivators SRC-1 and GRIP-1 [93]. Differential effects on ligand-induced coactivator binding to VDR have been reported for $1,25(OH)_2$-22-oxa-D_3 (OCT) as well [94]. While $1,25(OH)_2D_3$ could promote interaction of the VDR with SRC-1, TIF2 and AIB1, OCT supported only interaction with TIF2.

Issa *et al.* investigated 12 analogs for their abilities to mediate recruitment of the coactivators GRIP1 and RAC3 *in vitro* [95]. There was considerable ligand-specific variability in the strength of the VDR-coactivator interaction that was not correlated with the affinity of the ligands for the VDR.

Altered recruitment of coactivators by analog-activated VDR could provide a mechanism for gene-specific effects. Full activation (or repression) of gene transcription by the VDR may require the full complement of transcriptional components.

The AF-2 domain (helix 12) of the VDR is known to be critical for interaction with many of the components of the transcriptional complex, and mutations in this region abrogate the activity of $1,25(OH)_2D_3$ by influencing both heterodimerization with RXR and the coactivator binding [93,96]. Although these mutations do not affect VDR-RXR heterodimerization induced by the 20-epi-$1,25(OH)_2D_3$, they hamper SRC-1 binding and reduce transcriptional activity.

7. LIGAND-DEPENDENT VDR REGULATION

The liganded VDR has been shown to be much more resistant to intracellular degradation, as discussed above. Masuyama and MacDonald [97] demonstrated that the VDR is degraded primarily by the proteasome complex, a large macromolecular structure that degrades most cellular proteins. Inhibition of proteasome activity prevented VDR turnover and increased VDR content. However, control of VDR levels by vitamin D compounds appears to be complex, since ligands may not only stabilize the VDR, but also target the receptor for degradation.

There is evidence that ligands for steroid hormone receptors that dissociate more slowly have been shown to better protect the receptor from degradation [98]. Vitamin D analogs have differing abilities to stabilize the VDR, and in a few cases, this has been attributed to altered dissociation rates. The best-studied examples are the 20-epi compounds. Van den Bemd *et al.* [99] reported that KH1060 was much more effective than $1,25(OH)_2D_3$ in slowing the rate of VDR degradation in osteoblastic cells. Consistent with this was the report that while nuclear extracts of cells treated with the 20-epi analog CB1093 showed more VDRE binding than nuclear extracts from $1,25(OH)_2D_3$-treated cells, binding of the two vitamin D compounds to VDR

in vitro produced equivalent results in the EMSA. Thus, it appears that the higher potency of 20-epi analogs cannot be attributed directly to the distinct conformational change in the VDR, but rather to changes observed only in whole cells, most likely through the increased ability to stabilize the VDR.

Evidence that slowly dissociating vitamin D ligands are superior in stabilizing the VDR was reported by Peleg *et al.* [100]. They reported that the analog 1β-hydroxymethyl-3-epi-$1,25(OH)_2D_3$ is more sensitive to changes in the AF-2 domain, tends to dissociate more readily from the VDR, and is less potent than $1,25(OH)_2D_3$. Modifying this analog to contain 1β-hydroxymethyl and dimethyl groups at carbons 26 and 27 enhanced transcriptional activity, increased the stability of the VDR to proteolysis, and reduced the dissociation rate. Addition of a 16-ene to the molecule enhanced these properties even further.

Stabilization leads to an increase in VDR content in the cell, and this would largely explain the greater activity of certain analogs, notably those with the 20-epi configuration. Clearly, the degree of VDR up-regulation would be related to the activity of the degradative pathway in the cell. In cells with a very high rate of VDR turnover, ligand-dependent stabilization would produce a larger increase in VDR content. Thus, analogs with slow dissociation rates would be expected to be more active in these cells. This could provide a mechanism for cell-selective actions of vitamin D analogs.

A further complexity in VDR regulation was revealed in the recent report by Masuyama and MacDonald [97]. The VDR binds in a ligand-dependent manner to SUG1, a component of the proteasome complex. Overexpression of SUG1 increased VDR degradation and reduced $1,25(OH)_2D_3$-mediated activity. These results suggest that vitamin D ligand, by promoting interaction with SUG1, may target the VDR for degradation. The ligand specificity for SUG1 binding has not been rigorously examined, but since this interaction requires the AF-2 domain of the VDR [97], it is likely that analogs with altered interaction with this domain would differentially promote SUG1 binding. Thus, there appear to be counteracting effects of vitamin D compounds on VDR regulation, and the relative abilities of vitamin D compounds to stabilize the VDR or target the receptor for degradation may determine the VDR content, and hence biological activity, in a specific cell.

B. Interaction with the Serum Vitamin D Binding Protein (DBP) and Other Transporters

Vitamin D compounds are relatively hydrophobic with little solubility in aqueous solution and must be

transported *in vivo* attached to proteins. The major carrier of vitamin D compounds in the circulation is the serum vitamin D–binding protein (DBP). This protein binds all of the natural vitamin D metabolites with high affinity and circulates at a concentration of 5 µM, compared to picomolar levels of $1,25(OH)_2D_3$ (see Chapters 8 and 9). Other abundant proteins, such as albumin and lipoproteins, may bind lesser amounts of the natural vitamin D compounds, but with much lower affinity. However, they may play a role in transporting analogs that bind poorly to DBP. In the case of $1,25(OH)_2D_3$, over 99% is protein bound, mostly to DBP.

The structural elements of vitamin D compounds that affect DBP binding are different than for interaction with the VDR. The 1α-hydroxyl group is not required for DBP binding, and 1-desoxy compounds have a higher affinity than their 1-hydroxylated counterparts. On the other hand, DBP affinity is greatly affected by changes in the side chain, the most common site of modification in the therapeutically important vitamin D analogs. This altered DBP binding has been shown to play a critical role in the selectivity of several vitamin D analogs.

DBP performs two major functions with respect to vitamin D compounds: it enhances their circulating half-life and, importantly in the case of $1,25(OH)_2D_3$ and its analogs, it decreases tissue accessibility. In this way DBP acts as a reservoir and plays a key role in guarding against vitamin D intoxication. Thus, the DBP affinity will affect both the clearance rate and tissue uptake of vitamin D analogs.

Altered pharmacokinetics plays a central role in determining the unique biological profile of several analogs. The side chain modifications of most of the vitamin D compounds in development reduce DBP affinity and cause the analog to be rapidly cleared or poorly absorbed into the circulation. The best studied example of an analog that exerts its selectivity through this pharmacokinetic mechanism is OCT. The affinity of OCT for DBP is about 500 times lower than that of $1,25(OH)_2D_3$ [101]. As a result, this analog is cleared from the circulation more rapidly than $1,25(OH)_2D_3$ and achieves lower peak levels following injection as shown in Fig. 3, upper panel [102,103]. Despite the lower peak levels of OCT in the blood, the peak levels of the analog were greater than those of $1,25(OH)_2D_3$ in most target tissues, including the intestine as shown in Fig. 3, lower panel. This increased tissue content was short-lived, falling rapidly as the analog was cleared from the circulation. This "pulse" of OCT in the intestine elicited only a transient increase in calcium transport which fell to basal levels soon after OCT disappeared from the circulation (Fig. 4, upper panel) [103].
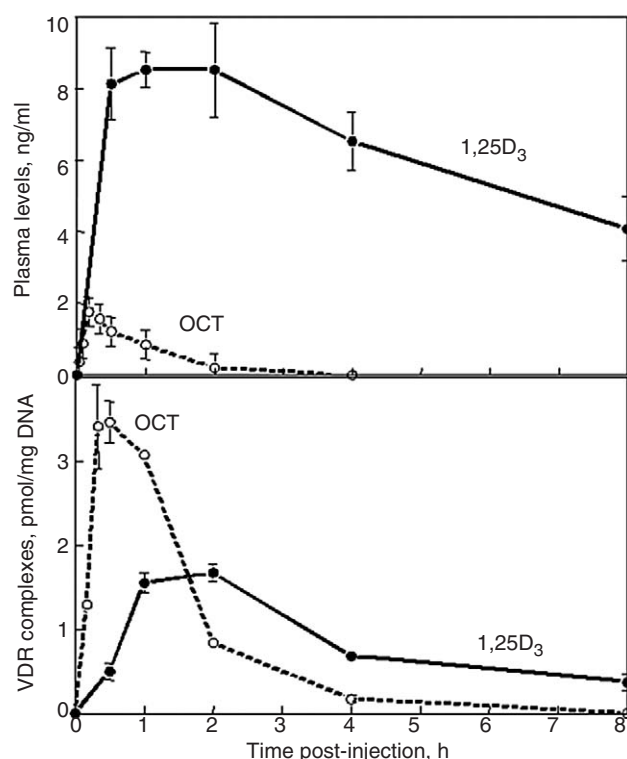

FIGURE 3 Plasma levels and intestinal localization of $[^3H]1,25(OH)_2D_3$ ($1,25D_3$) and $[^3H]OCT$ following a single intraperitoneal injection. Upper Panel: Plasma levels of HPLC-purified $[^3H]1,25(OH)_2D_3$ and $[^3H]OCT$ at various times postinjection. Lower Panel: Duodenal VDR content of $[^3H]1,25(OH)_2D_3$ and $[^3H]OCT$ at various time postinjection. From Brown *et al.* [103].

The effects of OCT on bone were also short-lived compared to those of $1,25(OH)_2D_3$ (Fig. 4, lower panel). Further evidence for a pharmacokinetics being responsible for the low calcemic activity of OCT were the sustained increases in intestinal calcium transport and bone mobilization with constant infusion of the analog, and the rapid return (within 24 hours) to baseline with cessation of the infusion [103].

OCT has been approved in Japan for treatment of secondary hyperparathyroidism in renal failure patients, and is in development for other applications including cancer and psoriasis. In parathyroid glands, OCT treatment produces a prolonged suppression of parathyroid hormone (PTH) gene transcription. The initial findings of Kobayashi *et al.* [102] indicated that injected $[^3H]OCT$ rapidly disappears from the parathyroid glands once the analog is cleared from the circulation, similar to the time course observed in the intestine and other tissues. However, the recent autoradiography study of Koike *et al.* [104] found that $[^3H]OCT$ persists in parathyroid cell nuclei. Furthermore, the nuclear binding of $[^3H]OCT$ in the parathyroid glands
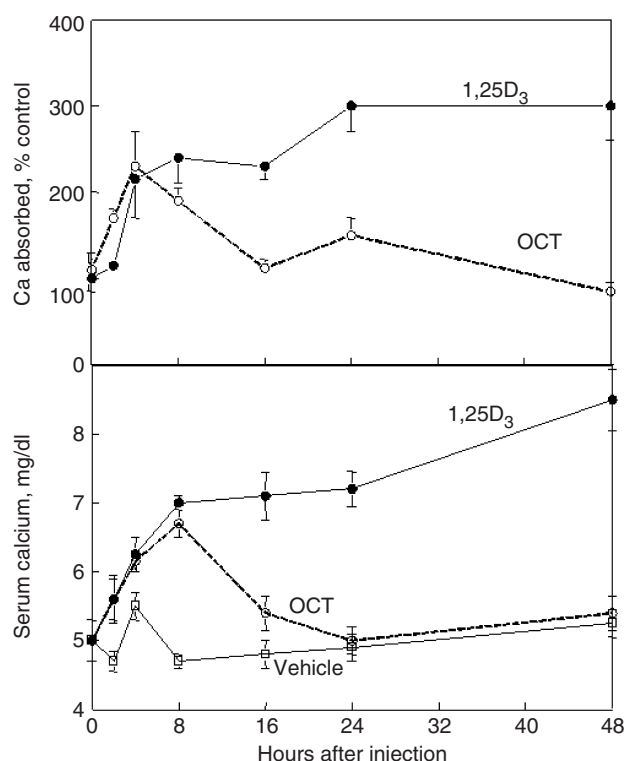
FIGURE 4 Effects of OCT and $1,25(OH)_2D_3$ on intestinal calcium transport and bone mobilization. Vitamin D–deficient rats were fed a 0.02% Ca diet for two days and then injected IP with 250 ng of $1,25(OH)_2D_3$ or OCT or vehicle. Intestinal calcium absorption was determined by the isolated duodenal loop method using ^{45}Ca. Bone calcium mobilization was estimated by the increase in serum calcium. From Brown *et al.* [103].

was saturated at a lower dose than the other tissues examined [61].

These findings would support a pharmacokinetic mechanism for the selectivity of OCT in the parathyroid glands. This rapidly cleared analog exploits the differences in the half-lives of the therapeutic response (prolonged PTH suppression) and the toxic responses (short-lived stimulation of intestinal calcium transport and bone mobilization). The molecular basis for the different durations of these responses is unclear. Enhancement of intestinal calcium transport and bone mobilization appear to require continuous exposure to vitamin D compounds, possibly because the inducible proteins mediating these response have relatively short half-lives. Alternatively, the transient responses could be due to a cessation of stimulation by OCT of the nongenomic pathway of vitamin D action as discussed below. The relatively long-lasting effects of all vitamin D compounds, including OCT, on PTH gene expression may be attributable to their slower metabolism in parathyroid cells as discussed in the next section.

The same pharmacokinetic mechanism may explain the effectiveness of OCT in the treatment of cancer. Abe *et al.* showed that OCT could effectively inhibit the growth of breast cancer cells in mice without producing hypercalcemia [30]. The short half-life of OCT did not prevent its beneficial antiproliferative activity. It is likely that this pharmacokinetic mechanism for analog selectivity will apply, with varying degrees, to all analogs with low DBP affinity. This mechanism may also apply to VDR ligands with nonvitamin D structures. Boehm *et al.* [105] screened a chemical library and found a nonsteroidal biphenyl compound that could activate the VDR. This compound was very potent in inhibiting cancer cell growth *in vitro*, but had very low calcemic activity *in vivo*. Further analysis showed that the new vitamin D ligand did not bind to DBP. Therefore, it is likely that the compound is rapidly cleared and unable to sustain calcemic effects in the intestine and bone.

On the other hand, analogs with higher DBP affinity than $1,25(OH)_2D_3$ tend to have longer circulating half-lives and less accessibility to target tissues. An example is ED-71 [2-(3-hydroxypropoxy)-$1,25(OH)_2D_3$], which is in development for osteoporosis [106] (see Chapter 85). This analog produces a smaller stimulation of bone mobilization and intestinal calcium transport than $1,25(OH)_2D_3$ due to its decreased uptake, but its calcemic effects are more prolonged due to its longer half-life. These altered pharmacokinetics may be responsible, in part, for the ability of ED-71 (but not $1,25(OH)_2D_3$) to produce net bone formation in ovariectomized rats.

It is important to note that there is a limit to the therapeutically beneficial effects of low DBP affinity. Analogs that bind extremely poorly to DBP may have little activity *in vivo*. An example of this is the analog $1,25(OH)_2$-24-trihomo-22-ene-D_3, which we tested for its suitability for secondary hyperparathyroidism (unpublished data). This compound was as potent as $1,25(OH)_2D_3$ in suppressing PTH in cell culture and had virtually no calcemic activity *in vivo*. However, the analog was virtually inactive in suppressing PTH *in vivo*. Subsequent characterization showed that the DBP affinity of this compound was more than 10,000 times lower than that of $1,25(OH)_2D_3$. It is likely that the analog did not achieve effective concentrations in the blood and was completely inactive *in vivo*. Therefore, DBP affinity is a critical parameter that requires testing early in the development of therapeutic vitamin D analogs.

A second consideration with respect to DBP affinity is its influence on analog activity in cells cultured in serum-containing medium. Analogs with lower DBP

affinity than $1,25(OH)_2D_3$ will be taken up more readily in the presence of serum and will be more active under these conditions. This is often the explanation for why some analogs have higher potency than predicted by their VDR affinity. A number of studies have documented this serum effect on the relative activities of $1,25(OH)_2D_3$ and its analogs [107–109]. Correct interpretation of the potencies of vitamin D analogs will require the determination of DBP affinity.

Another consequence of low DBP binding is the freedom of the analogs to associate with other serum proteins. This could potentially alter the delivery of vitamin D analogs to various target tissues. Currently, there is considerable disagreement as to the degree of binding of vitamin D metabolites and analogs to the various potential carriers. Okano *et al.* [101] and Kobayashi *et al.* [110] reported differences in the association of 22-oxa-l,25(OH)_2D_3 and 1,25-(OH)_2D_3 with serum lipoproteins. When 22-oxa-l,25(OH)_2D_3 was mixed *in vitro* with human plasma, it bound almost exclusively (99%) with the lipoprotein fraction, mainly with chylomicrons and low density lipoproteins (LDL), whereas less $1,25(OH)_2D_3$ was bound to lipoprotein (60%). A similar study by Teramoto *et al.* [111] reported much less binding of these two compounds to lipoprotein fractions. The significance of the differences in lipoprotein binding in the selectivity of 22-oxa-l,25(OH)_2D_3 is unclear, as the role of carrier proteins in the delivery of vitamin D compounds has received only slight attention [112–115]. At present, a comparison of binding of 22-oxa-1,25(OH)_2D_3 and 1,25(OH)_2D_3 to lipoproteins has been done only in human plasma and only *in vitro.* Therefore, the relevance to ligand selectivity observed in experimental animals is not clear. Better evidence for a mechanism for selectivity involving carrier protein distribution would be the demonstration of differences in the binding of vitamin D analogs and 1,25(OH)_2D_3 to rat lipoproteins *in vivo.*

C. Cellular Metabolism by the Vitamin D-24-hydroxylase and Other Enzymes

Target cell metabolism has been shown to play an important role in several steroid hormone systems [116]. In mineralocorticoid-responsive tissues, the receptor that mediates the action of aldosterone binds glucocorticoids with equal affinity. Selectivity is achieved in these tissues by efficient degradation of glucocorticoids, rendering them unavailable to the receptor. Alternatively, cells can convert hormones to more active metabolites as in the conversion of T_4 to T_3 or estrone (E_1) to estradiol (E_2).

Vitamin D compounds are metabolized primarily, but not exclusively, by the vitamin D-24-hydroxylase, which catalyzes a series of oxidations at carbons 24 and 23 in the side chain of the molecules (see Chapters 6 and 80). Oxidative cleavage between C23 and C24 yields calcitroic acid, probably via an aldehyde intermediate. The 24-hydroxylase is highly induced by $1,25(OH)_2D_3$ and its analogs, and it is generally believed that side chain metabolism has an attenuating effect on vitamin D action. Intermediary metabolites of $1,25(OH)_2D_3$ produced by this pathway have lower VDR affinity and are usually less active than the parent, but exceptions have been noted and are discussed below. Since the most common site of modification of vitamin D analogs is in the side chain, it would be expected that their metabolism would differ from that of $1,25(OH)_2D_3$. In fact, differences in the rates of catabolism and the end-products of metabolism have been shown to account, at least in part, for the unique properties of several vitamin D analogs.

Analogs that are quickly degraded within target cells would have lower overall biological activity. This effect was illustrated in a study by Zhao *et al.* in cell culture [117]. They examined the antiproliferative effects of $1,25(OH)_2D_3$ and several analogs on cultured MCF-7 breast cancer cells (Fig. 5). The ED50 values for each analog were reduced by cotreatment with ketoconazole, a cytochrome P450 inhibitor that blocks 24-hydroxylase activity. The higher ED50 in the absence of the inhibitor was due to the influence of target cell catabolism of the vitamin D compounds. The degree of the reduction of the ED50 by ketoconazole cotreatment varied for each analog, likely due to the differential rates of catabolism of the compounds. Other examples of analogs whose activity may be influenced by their catabolic rate include EB1089 and 20-epi-1,25(OH)_2D_3. These compounds have higher biological activity than $1,25(OH)_2D_3$ despite similar VDR affinities. The disparity has been attributed, at least in part, to their very slow rates of intracellular catabolism [108,118–120]. The recent report of Lin *et al.* showed that EB1089 produced the same changes in gene expression as $1,25(OH)_2D_3$ in squamous carcinoma cells, but that the effects were more prolonged [121]. The difference was abolished when $1,25(OH)_2D_3$ was co-incubated with ketoconazole. It is important to note that rapid disappearance of an analog in the target cell could produce the same differential effects as accelerated clearance as described for analogs with low DBP affinity.

The selectivity of vitamin D analogs *in vivo* may also be attributed to cell-specific differences in the rates of catabolism of an analog. For example, OCT
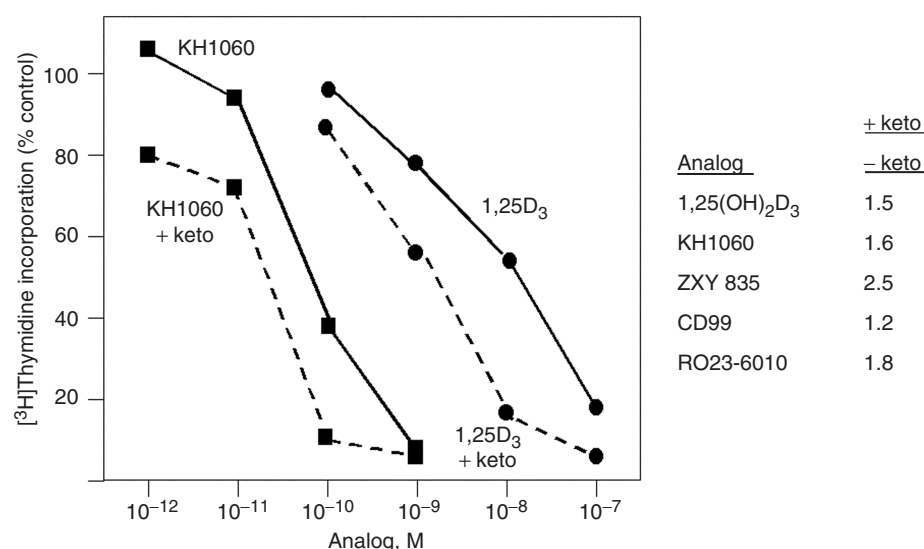
FIGURE 5   Influence of catabolism on the biological activities of vitamin D analogs. Left Panel: The antiproliferative effects of $1,25(OH)_2D_3$ and its analog KH1060 on MCF-7 breast cancer cells was measured in the absence or presence of the cytochrome P450 inhibitor ketoconazole. Blocking the catabolic enzyme (24-hydroxylase) enhanced the antiproliferative activities. Right Panel: The ratio of the ED50 in the presence (+keto) versus absence (−keto) of ketoconazole. The activity is enhanced by ketoconazole in all cases, but the variation between analogs likely reflects differences in their rates of catabolism. From Zhao *et al.* 1996 [117].

appears to be degraded at the same rate as $1,25(OH)_2D_3$ in parathyroid cells [122], more rapidly in keratinocytes [123] and more slowly by monocytes [124]. The rates of catabolism are consistent with the similar activities of OCT and $1,25(OH)_2D_3$ in parathyroid cells [122] and lower activity of OCT in keratinocytes [123]. The explanation for this cell-specific catabolism is unclear, but certainly deserves further study since exploitation of the catabolic differences between vitamin D compounds could produce highly selective therapeutic agents.

There are also differences in the amount of catabolic activity in various cell types. In the study by Zhao *et al.* described above [117], ketoconazole had no effect on the activities of the analogs in the osteoblastic cell line MG-63, indicating that the catabolic activity in these cells is very low. This variation in catabolic activity could lead to cell-specific differences in the actions of $1,25(OH)_2D_3$ and its analogs. In cells with high catabolic activity, analogs that are better substrates for the 24-hydroxylase would be rapidly degraded and less active, whereas ligands that are poor substrates would be more active. By this mechanism alone, each analog could have a unique biological profile.

Structural modifications in some vitamin D analogs prevent the completion of the side chain cleavage pathway and yield stable active intermediates that may accumulate in target cells. One example is $1,25$-$(OH)_2$-16-ene-D_3. This analog is catabolized by the

24-hydroxylase to the 24-oxo intermediate [125], but further oxidation occurs very slowly. The $1,25(OH)_2$-16-ene-24-oxo-D_3 retains significant biological activity *in vitro* [126] and *in vivo* [45]. In a murine model of autoimmune encephalitis, $1,25(OH)_2$-16-ene-24-oxo-D_3 was as active as $1,25(OH)_2$-16-ene-D_3 and $1,25(OH)_2D_3$ in suppressing the immune response, but it was substantially less calcemic. A similar interruption in side chain catabolism was noted for 20-epi-$1,25(OH)_2D_3$. The 24-oxo metabolite of this analog accumulates in target cells and may be responsible in part for the high biological activity of the parent 20-epi compound [108]. KH1060, which has been shown to be much more active than $1,25(OH)_2D_3$ in slowing cell proliferation, is rapidly metabolized to 24- and 26-hydroxylated metabolites that are also more potent than $1,25(OH)_2D_3$ [127]. These two metabolites have potencies similar to the parent KH1060 in the induction of osteocalcin expression by osteoblast-like cells, in enhancing VDR stability by increasing resistance to proteolysis, and in binding of the VDR to the osteocalcin VDRE [128]. Thus, the 24- and 26-hydroxy metabolites contribute significantly to the overall effects of the parent KH1060. Komuro *et al.* [129] found that $1,25(OH)_2$-26,27-F_6-D_3 is converted to $1,23,25(OH)_3$-26,27-F_6-D_3, which is resistant to further metabolism and retains significant activity.

Other metabolic enzymes may play a key role in analog selectivity. Reddy and his colleagues recently

discovered that $1,25(OH)_2D_3$ can undergo epimerization at carbon 3 to change the 3β-hydroxyl to the 3α configuration [130]. The enzyme responsible for 3-epimerization is not ubiquitously expressed. This reaction has been documented in keratinocytes, parathyroid cells, osteoblastic cells, and colon cancer (Caco-2) cells, but not by the kidney or myeloid leukemia (HL-60) cells. In parathyroid cells, 3-epi-$1,25(OH)_2D_3$ has nearly the same potency as $1,25(OH)_2D_3$ in suppressing PTH, but is catabolized more slowly by the 24-hydroxylase and may accumulate in these cells [Brown, 1999 #22670]. The slower inactivation, if it occurs in parathyroid glands *in vivo*, could be responsible, at least in part, for the prolonged effects of $1,25(OH)_2D_3$ on PTH secretion.

An example of cell-specific metabolism was reported by Kamao *et al.* [131]. OCT was metabolized mainly by the 24-hydroxylase in intestinal (Caco-2) cells and renal (LLC-PK1) cells, whereas in osteoblastic (UMR-106) cells, OCT was metabolized by the 3-epimerization and 25-dehydration pathways. Differences between $1,25(OH)_2D_3$ and its analogs in their relative rates of metabolism through these pathways could lead to preferential action in one cell over the other.

The structural requirements for substrates of the enzyme responsible for 3-epimerization is under investigation. Reddy *et al.* found that the rate of 3-epimerization of $1,25(OH)_2$-16-ene-23-yne-20-epi-D_3 10 times faster than for $1,25(OH)_2$-16-ene-23-yne-D_3 [132]. It is not clear if the 20-epi modification facilitates 3-epimerization of other analogs as well, but structural alterations that facilitate the conversion to the 3-diastereomer may further slow the rate of metabolism in cells with high 3-epimerase activity and therefore enhance the analog action in a cell-specific manner.

D. Nongenomic Activity Mediated by a Cell-surface Receptor

Vitamin D compounds can activate a number of signaling pathways, perhaps by activating a distinct receptor at the plasma membrane [1,82,133] (see Chapter 23). These effects are observed within seconds to minutes following exposure to $1,25(OH)_2D_3$, too quickly to involve changes in gene transcription. One of the best characterized rapid actions is the *ex vivo* stimulation of calcium movement across the intestinal epithelium, a process termed *transcaltachia*. Nemere and co-workers perfused chick duodenum with $1,25(OH)_2D_3$ and observed increased calcium movement from the lumen to the perfusate within minutes [134]. Others have noted rapid stimulation by $1,25(OH)_2D_3$ of PKC activation and translocation, phosphate fluxes, alkaline phosphatase,

cGMP, and phosphoinositide metabolism in cell culture. $1,25(OH)_2D_3$ increases cytosolic calcium within minutes in a number of cell types, including osteoblasts, parathyroid cells, human myeloid leukemia cells, enterocytes, and myocytes. $1,25(OH)_2D_3$ has been shown to rapidly activate mitogen-activated protein kinase (MAPK) [135,136] and stimulate opening of chloride channels [137].

The nature of the receptor(s) that mediates these rapid actions is unclear. Early evidence suggested that the rapid effects of calcitriol may be mediated by the VDR. $1,25(OH)_2D_3$ cannot rapidly increase cGMP in skin fibroblasts from patients with vitamin D–resistance rickets that lack a functional VDR. In addition, calcitriol has also been shown to stimulate phosphoinositide metabolism in isolated enterocytes from adult rats, but not in those from 16-day-old rats that do not express intestinal vitamin D receptors [138]. However, the finding that $1,25(OH)_2D_3$ cannot stimulate transcaltachia in vitamin D–deficient chick duodenum suggested that this process, and perhaps other nongenomic actions, requires vitamin D–inducible gene products.

Evidence now indicates that nongenomic responses may be carried out by a distinct receptor [139–141]. The ligand specificity for the rapid actions is different than that for the genomic response [142–144]. Stimulation of the nongenomic actions does not require the 1α-hydroxyl group; $25(OH)$-23-yne-D_3 and its 16-ene counterpart can stimulate transcaltachia, but do not bind the VDR [142]. On the other hand, $1,25(OH)$-16-ene-23-yne-$_2D_3$ and calcipotriol, which have high VDR affinity, do not produce transcaltachia [142]. Further evidence for the distinct nature of the nuclear and membrane receptors is the finding that OCT has genomic, but not nongenomic activity, in rat osteoblastic cells [145]. More recent studies have demonstrated that vitamin D analogs that are locked in the 6-cis configuration act only as agonists for the putative membrane receptor and do not bind to the VDR [143].

The functions of the nongenomic actions of $1,25(OH)_2D_3$ in most cell types and their relevance *in vivo* are still unclear. In the intestine, it is well established that exposure of the basolateral membrane to $1,25(OH)_2D_3$ stimulates transcaltachia [142]. In chondrocytes, nongenomic actions of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ alter membrane lipid turnover, prostaglandin production, and protease activity that lead to modification of bone matrix and calcification [146]. In other cells, the nongenomic events have been proposed to modulate the genomic actions of $1,25(OH)_2D_3$ [147,148], but this remains controversial. Numerous studies have presented evidence that the nongenomic actions may not be critical for $1,25(OH)_2D_3$-mediated gene activation [73,149–152] or inhibition of cell

proliferation [151,153]. However, nongenomic stimulation of protein kinases could potentially influence the VDR-mediated effects of $1,25(OH)_2D_3$. As introduced earlier, genomic activity of the VDR is decreased by PKC, which is stimulated by $1,25(OH)_2D_3$ activation of the membrane receptor. Thus, differential effects of vitamin D analogs on the membrane receptor could influence its genomic actions as well as the VDR-independent effects of the signaling pathways.

The low calcemic activity of two analogs, $1,25(OH)$-16-ene-23-yne-D_3 and calcipotriol, could be due to their inability to stimulate transcaltachia in the intestine [142]. Several other analogs, including 1,25,28-trihydroxyvitamin D_2, $1,25(OH)_2$-24-dihomo-22-ene-D_3, $1,25(OH)_2$-24-trihomo-22-ene-D_3, that stimulate genomic responses (calbindin D9k) in the intestine but not calcium transport [53,54] may lack nongenomic activity, but these analogs have not been tested. On the other hand, OCT has been shown to stimulate transcaltachia in the intestine [145]. The inability of OCT to sustain a high rate of calcium transport after its disappearance from the circulation (Fig. 3) could be due to the loss of stimulation of the nongenomic pathway(s).

The role of the nongenomic pathway in bone is even less clear. As described above, the nongenomic effects of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ may play a role in bone formation [146]. $1,25(OH)_2D_3$ is known to stimulate both the production and phosphorylation of an osteoblast-derived protein, osteopontin. Safran *et al.* [154] found that the analog 25(OH)-16-ene-23-yne-D_3, which is known to stimulate only the nongenomic pathway (no VDR affinity), cannot induce osteopontin but does promote its phosphorylation. Clearly, non-genomic activities could play a role in the vitamin D effects on bone formation. On the other hand, the role of nongenomic effects on bone resorption is not known. It is possible that analogs that are incapable of activating or sustaining a nongenomic response are unable to efficiently induce or maintain bone mobilization.

A plasma membrane protein from chick duodenum was isolated that binds vitamin D analogs with affinities that correlate with their activation of transcaltachia [139]. Antibodies raised against the protein recognize primarily a 66 kDa peptide by immunoblot analysis [140]. Most importantly, this antibody inhibits the ability of $1,25(OH)_2D_3$ to stimulate PKC activity in chondrocyte membranes. However, this protein has not been identified or cloned.

Another membrane receptor has been identified in ROS 24/1 rat osteosarcoma cells that do not express the VDR [141]. A plasmalemma protein of approximately 36 kDa was shown to be covalently cross-linked to 3-bromoacetylated $1,25(OH)_2$-[^{14}C]D_3. The labeled protein was isolated by isoelectric focusing, and a partial sequence identified it as annexin II. Polyclonal antibodies to annexin II decreased binding of $1,25(OH)_2$-[^{14}C]D_3 and blocked the increase in cytosolic calcium by $1,25(OH)_2D_3$ [155]. The role of annexin II as a receptor for vitamin D analogs is unclear.

The difference in the sizes of the chick duodenal membrane receptor and annexin II suggests that there may be multiple membrane receptors for $1,25(OH)_2D_3$ that are structurally and functionally distinct. The relative ligand specificities and tissue localization of the receptors are not yet known. Despite questions concerning the physiologic relevance of the nongenomic pathways, these membrane-binding sites could potentially offer new pharmacologic targets that could produce cell-specific or even process-specific effects of vitamin D analogs.

E. Intracellular Binding Proteins

Proteins of the heat shock-70 family are now known to bind vitamin D compounds and appear to play a determining role in their metabolism and actions [156–163]. These proteins, discovered during the investigation of the vitamin D resistance observed in new world primates, were found to be overexpressed in a marmoset B-lymphoblast cell line B95-8. Unfractionated B95-8 cytosol containing a mixture of intracellular vitamin D–binding proteins (IDBPs) was used in a competitive binding assay with [^{3}H]25(OH)D_3 to assess binding affinities of various vitamin D ligands. 25(OH)D_3 and 25(OH)D_2 bound most avidly. $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ had about $^1/_3$ the affinity of 25(OH)D_3, and 1α(OH)D_3, which lacks a 25-hydroxyl group, did not bind at all [156,160]. Overexpression of IDBP-1 stimulated the transactivation by $1,25(OH)_2D_3$ [161] and enhanced the conversion of 25(OH)D_3 to $1,25(OH)_2D_3$ [162]. More recently, it was reported that IDBP-1 predominantly directs delivery of ligands to the VDR, while IDBP-3 facilitates mitochondrial delivery of substrates via an N-terminal targeting signal for the inner mitochondrial membrane where it interacts directly with the vitamin D-1α-hydroxylase [163]. It would seem likely the mitochondrial delivery by IDBP-3 of active vitamin D compounds (i.e., VDR ligands) would accelerate their metabolism and inactivation. A more thorough discussion of the IDBP and their roles in vitamin D metabolism and action can be found in Chapter 21.

Only a few vitamin D analogs have been tested for their binding to the IDBPs. Calcipotriol, EB1089, and KH1060 were found to have no apparent binding affinity for the IDBPs in unfractionated supernatants from B95-8 cells [159] when competing with [^{3}H]25(OH)D_3

as ligand. These three analogs are modified in their side chains, indicating the importance of this portion of the vitamin D structure in binding to the IDBPs. In addition, the initial studies used unfractionated cytosol as a source of a mixture of IDBPs; the interaction of the analogs with the individual IDBPs has not been reported.

The finding that distinct IDBPs may be involved in enhancement of transactivation and the facilitation of metabolism suggest a complex means for potentially affecting analog selectivity. High affinity binding to IDBP-1 would appear to increase the potency, whereas binding to IDBP-3 would facilitate metabolic inactivation. Relative differences in the interactions of analogs in binding to the IDBPs along with cell-specific differences in the relative expressions of the IDBPs could contribute to the unique biological profiles of the analogs. Future studies are required to assess the impact of the IDBPs on the actions of $1,25(OH)_2D_3$ and its analogs.

III. CONCLUDING REMARKS

Discovery of the nonclassical actions of $1,25(OH)_2D_3$ has spawned new interest in vitamin D therapy for the treatment of hyperproliferative diseases such as cancer and psoriasis, and endocrine disorders including hyperparathyroidism, as well as for immunosuppression to prevent autoimmunity and transplant rejection. Hypercalcemia has limited or precluded the use of $1,25(OH)_2D_3$ for most of these applications. Several new vitamin D analogs that retain the desired therapeutic activity but with less toxic (calcemic) side effects have now reached the marketplace. Calcipotriol is now available for psoriasis, and three analogs, 19-nor-$1,25(OH)_2D_2$, OCT, and $1\alpha(OH)D_2$, have been approved for secondary hyperparathyroidism. Many other analogs are in development for other applications. Perhaps the most critical of these is cancer.

The current approach for developing therapeutic vitamin D analogs involves brute force testing of hundreds if not thousands of compounds. A more thorough understanding of the factors that determine the biological profile of an analog could greatly reduce development time. Our current knowledge of vitamin D physiology and biochemistry indicates that the overall activities of vitamin D compounds are determined by the combined interactions with several key proteins: the nuclear vitamin D receptor, the serum vitamin D binding protein, the 24-hydroxylase, and a membrane receptor. A major goal is to understand how the integrated interactions lead to the *in vivo* actions of the analogs. This requires an understanding of both the

structure-activity relationships for each of these interactions and the role that these proteins play in the desired and undesired activities. As our knowledge of these two areas increases, so does the reality of designing vitamin D analogs with precise target specificity.

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Molecular Basis for Differential Action of Vitamin D Analogs

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I. INTRODUCTION

The most prominent physiological role of the hormonally active metabolite of vitamin D_3, $1\alpha,25$-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], is the regulation of calcium and phosphorous homeostasis as well as bone remodeling through its actions in the intestine, kidney, and bone [1–4]. The hormone also contributes to growth and differentiation of epidermal cells [5] and the bone marrow precursors of osteoclasts [6–9]. As discussed in detail in subsequent chapters, at pharmacological concentrations $1,25(OH)_2D_3$ also has immunoregulatory effects [10] and induces differentiation and inhibits growth of psoriatic skin [11] and a variety of malignant cell types [12–17].

Genomic and nongenomic signal transduction pathways are believed to mediate the diverse effects of this hormone. The genomic pathway has been well established and is mediated by the nuclear vitamin D receptor (VDR) [18]. The VDR belongs to a large family of transcription factors that contain two highly conserved zinc-binding finger structures in their DNA binding domain [19]. Transcriptional activity of the VDR depends primarily on its binding with $1,25(OH)_2D_3$ [20]. This interaction induces conformational changes in the receptor [20,21] and facilitates heterodimerization with the retinoid X receptor (RXR) [22–24], promotes binding to specific DNA responsive elements [23,25,26], causes recruitment of transcription coactivators of the p160 family and other components of the transcription apparatus, through interaction with the DRIP complex, all of which eventually lead to regulation of gene expression [27–31].

Several lines of evidence suggest that the broad range of physiological and pharmacological activities induced by $1,25(OH)_2D_3$, both those related to regulation of calcium metabolism and those associated with the regulation of growth and differentiation, are mediated by transcriptional activities of VDR. For example, in hereditary vitamin D–resistant rickets (HVDRR), defects in VDR cause severe bone disease and deficient calcium absorption [20]. In the same disease, the growth inhibitory effect of $1,25(OH)_2D_3$ on lectin-induced T-cells is diminished [32]. Finally, a link between normal development of hair follicles and functional VDR is suggested by the frequent presence of hairlessness (alopecia) in rickets patients with defective VDR [20]. Similar phenotypes are found in VDR-ablated mice, confirming the role of VDR in development of bone disease and alopecia [33–35]. In cell culture systems, the growth regulatory responses to $1,25(OH)_2D_3$ are correlated with the levels of VDR [36]. In the heterogeneous human myeloid leukemia cell line HL-60, $1,25(OH)_2D_3$ regulates the growth of clones with high VDR levels, but clones with few or no VDRs do not respond to $1,25(OH)_2D_3$ [37]. In animal models, growth inhibition is induced by $1,25(OH)_2D_3$ only in solid tumors containing VDR [17]. Additional support for the potential role of the VDR in cellular growth and differentiation is suggested from the abnormal development of mammary glands in VDR-null mice. In the same animals, there is also an increase in susceptibility of skin to carcinogen-induced tumorigenesis and hyperplasia of epithelial cells lining the colon [38–40].

In conclusion, VDR appears to be essential for both physiological and pharmacological responses to $1,25(OH)_2D_3$.

The wide range of responses transduced through the VDR provide the organic chemist and the biologist with the challenge of developing drugs that enhance or diminish specific VDR-mediated responses, and which might eventually be useful for treatment of disease. This chapter focuses on the molecular mechanisms by which chemically and stereochemically modified analogs of $1,25(OH)_2D_3$ produce different effects with respect to activation of the VDR.

Affinity and transcriptional activity

Catabolism & side-chain orientation

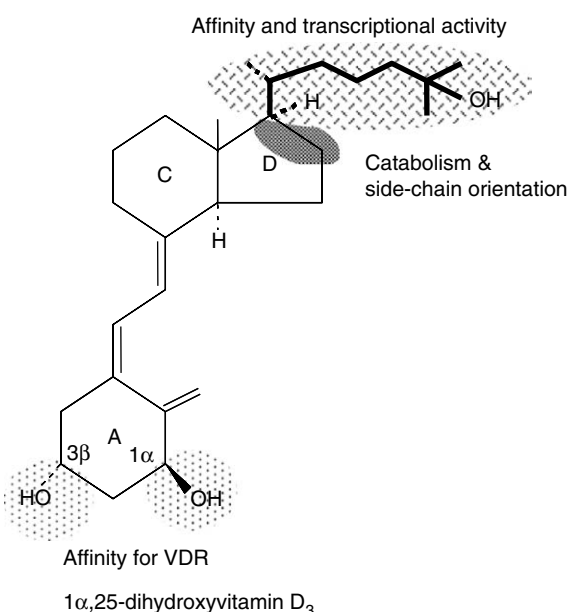Affinity for VDR

1α,25-dihydroxyvitamin D₃

FIGURE 1 Structural formula of $1,25(OH)_2D_3$. Shaded areas indicate structural features that regulate ligand binding and transcriptional activity of VDR.

II. STRUCTURAL REQUIREMENTS FOR TRANSACTIVATION OF THE VDR BY ITS NATURAL LIGAND, 1,25(OH)₂D₃

$1,25(OH)_2D_3$-mediated transcriptional activity of VDR depends primarily on the following three structural features of the ligand: the A-ring, the side chain, and the D-ring (Fig. 1) [41]. The structural features of the ligand that are important for binding to VDR were initially elucidated through studies of naturally occurring metabolites of vitamin D_3 and synthetic analogs of $1,25(OH)_2D_3$ [41,42]. Recent studies on the crystal structure of the VDR bound to its natural hormone confirm the earlier studies and demonstrate how these structural features interact with specific amino acid residues in the ligand-binding pocket and how these interactions contribute to tight binding and to conformational changes in the ligand-binding domain (LBD) that lead to transcriptional activation of the VDR [43].

The LBD of VDR (like that of other steroid receptors) is multifunctional: it regulates dimerization [29], interaction with transcription factors, and general transcriptional activity [28–31]. The structure of VDR's LBD is similar to that of other ligand-binding nuclear receptors in that it consists of 12 alpha helices organized in an antiparellel "sandwich" [43]. At the extreme C-terminus of the VDR is helix 12, a highly conserved sequence that contributes the core for the transcription activation function of the LBD that is called *activation function 2* (*AF-2*) [44,45]. $1,25(OH)_2D_3$ binding to VDR causes significant structural changes that decrease the sensitivity of the LBD to proteolytic digestion. These changes allow the VDR to form high-affinity interactions with other proteins, including the RXR, transcription coactivators of the p160 family (such as the steroid receptor coactivator 1 and the glucocorticoid receptor-interacting protein) and bridging factors such as the vitamin D receptor-interacting protein (DRIP 205) [28–30] (Fig. 2). These interactions are dependent on the availability of residues in the helix 12/AF-2 core for interaction with these proteins.
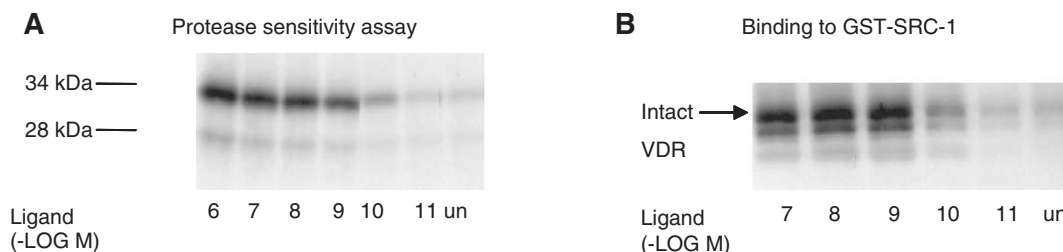

FIGURE 2 Correlation between stabilization of VDR against proteolytic digestion and its ability to interact with the nuclear receptor interacting domain of the steroid receptor coactivator 1 (SRC-1). (**A**) *In vitro* synthesized ^{35}S-labeled human VDR was incubated with the indicated concentrations of $1,25(OH)_2D_3$ and then subjected to limited proteolytic digestion by trypsin. The proteolytic products were separated by SDS-PAGE and detected by autoradiography. (**B**) Binding of ^{35}S-labeled synthetic human VDR to the fusion protein GST-SRC-1 was assessed by pull-down assay. The synthetic VDR was incubated with the indicated concentrations of $1,25(OH)_2D_3$ and with recombinant GST-SRC-1 and glutathione-Sepharose beads. The VDR bound to SRC was eluted and separated by SDS-PAGE. The amount of bound VDR was assessed by autoradiography of the gels. Note the correlation between ligand-dependent stabilization of VDR conformation and its ability to interact with SRC-1 [135].
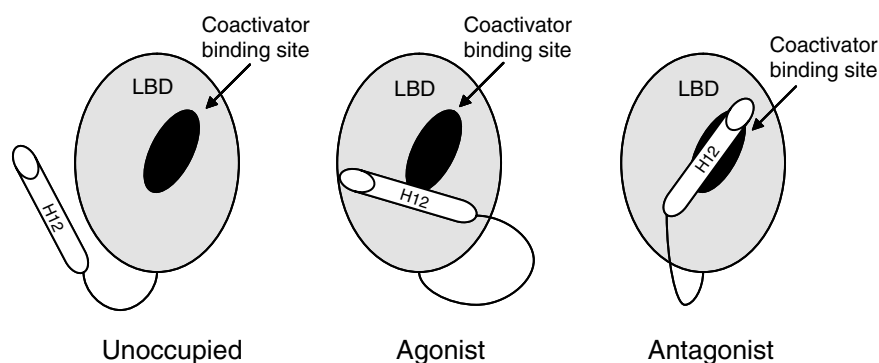
FIGURE 3 Schematic illustrations of the hypothetical conformations of unoccupied, agonist-occupied, and antagonist-occupied VDR LBD. Note the change in the position of helix12/AF2 core with respect to the coactivator binding site. In the unoccupied LBD, helix 12 is extended, and its residues are not associated with the LBD. In the agonist-occupied LBD, helix 12 changes position and is brought near the coactivator binding site. In the antagonist-occupied LBD, helix 12 masks the coactivator binding site and so prevents interaction with transcription coactivators. The conformations of unoccupied and antagonist occupied LBDs were adapted from structural studies of other LBDs [49].

The conformation of the AF-2 core is different in unoccupied, agonist-occupied and antagonist-occupied LBDs of other nuclear receptors (such as the estrogen receptor or progesterone receptor), and these differences have a strong effect on the target-tissue preferences and gene-regulatory events of the various ligand-receptor complexes (Fig. 3) [46–49]. Although only the structure of the agonist-bound VDR LBD has been published so far, we hypothesize that similar changes probably occur in the unoccupied VDR once it binds to agonists or antagonists.

A. The A-ring

The backbone of $1,25(OH)_2D_3$ is similar to that of cholesterol and the steroid hormones except that the four-ring structure has been disrupted by ultraviolet irradiation of the second (B) ring between carbons 9 and 10 (Fig. 1) [2–4]. This change makes the already flexible organic compound even more flexible, giving the A-ring the freedom to rotate from the steroid-like ("folded") conformation to a vitamin ("extended") conformation [50]. It has been shown that the steroid-like conformer does not interact with VDR, and therefore it was suggested that the vitamin conformer should be the one to bind with high affinity to the VDR [50]. However, the crystal structure of the VDR LBD showed that the position of the A-ring in the binding pocket is in between the cis and trans conformations, thus suggesting that modifications that restrict the flexibility of the A-ring may compromise ligand binding to the VDR.

The high affinity of vitamin D metabolites and analogs for the VDR also depends on the hydroxyl groups in the A-ring, particularly the 1α-hydroxyl [41,42]. For example, the precursor of $1,25(OH)_2D_3$, 25-hydroxyvitamin D_3, binds poorly to VDR, but hydroxylation of this metabolite at the 1α position induces high affinity for the receptor [41]. Likewise, another natural metabolite of vitamin D_3, 24,25-dihydroxyvitamin D_3, also binds poorly to the VDR, but its 1α-hydroxyl metabolite has a significantly greater affinity [51]. Similarly, $1,25(OH)_2D_3$ analogs that have modified side chains and lack the 1α-hydroxyl group bind poorly to VDR, whereas addition of hydroxyl or fluoride groups restores binding to VDR [52]. The explanation for these requirements is provided by X-ray crystallography of the LBD, which shows that the natural hormone is anchored in the binding pocket with its A-ring facing the interior and its 1α-hydroxyl group forming hydrogen bonds with arginine 274 and serine 237 [43]. In addition, although structure-function studies show that the hydroxyl group at the 3β position is less important for high affinity binding to the VDR, this hydroxyl group is also forming hydrogen bonds, one with serine 278 and the other with tyrosine 143. This structural information has been confirmed by laboratory-made or natural mutations in the hVDR. The latter is the R274L mutation that causes almost complete loss of $1,25(OH)_2D_3$ binding to the VDR and also causes hereditary vitamin D–resistant rickets [53]. The structural information has also allowed the manipulation of vitamin D analogs' affinity for the VDR by introducing chemical modifications that provide additional anchoring points for the A-ring

in the binding pocket. The most notable modifications are in C-2, and they include 2α-methyl, 2α-alkyl, and 2α-hydroxyalkyl analogs [54].

B. The Side Chain

The ability of $1,25(OH)_2D_3$ to bind, and more importantly to transactivate, the VDR depends on 25-hydroxylation of the side chain. This is proven by the fact that the synthetic prehormone 1α-hydroxyvitamin D_3 cannot bind or transactivate the VDR *in vitro* or in cell culture. Again, structural resolution of the VDR bound to the natural hormone [43] shows that the 25-hydroxyl group forms two hydrogen bonds, one of which (histidine 397) is essential for tight binding of the ligand to the VDR, and the other (histidine 305) is essential for stabilization of the VDR in transcriptionally active conformation. The side chain of $1,25(OH)_2D_3$ is facing the C-terminal region of the VDR and forms contacts with helix 11 and helix 12/AF-2 core. The AF-2 core contains residues that are essential for binding

to coactivators and for assembly of the AF-2 in transcriptionally active conformation. Therefore, it is likely that modifications in the side chain have the potential to alter transcriptional activation of the VDR either by contributing to ligand affinity for the VDR or by changing the conformation of AF-2 to increase or decrease its transcriptional activity. Because certain side chain modifications increase transcriptional activity of the VDR a 100- to 10,000-fold without changing affinity (Table I) suggests that these two functions of the side chain are independent. For example, transcriptional superagonists can be generated by epimerization of carbon 20 [55]. In the 20-epi analogs, this stereochemical change slightly reduces affinity for VDR, but it increases the potency of VDR-mediated transcriptional activity more than a hundredfold [55]. Additional evidence that the side chain contribution to VDR's transactivation is a separate function from its contribution to ligand affinity for the VDR is provided by studies with hybrid analogs (Table I). These analogs contain A-ring modifications that compromise affinity for the VDR significantly, but in the presence of a potent side chain,

TABLE I   Structure-functions Relationships of 1α,25-Dihydroxyvitamin D_3 and Its Analogs

Ligand formula	Receptor binding[a]	Transcription[b]
Parent compound		
$1α,25(OH)_2D_3$	1×10^{-9}	2×10^{-9}
A-ring modified analogs		
$25\text{-OH-}D_3$	1.4×10^{-7}	0.5×10^{-7}
$1β,25(OH)_2D_3$	2×10^{-7}	2×10^{-7}
$1β\text{-(hydroxymethyl)-}3α\text{-25-OH-}D_3$	8×10^{-8}	$>10^{-6}$
Side chain-modified analogs		
$1α\text{-OH-}D_3$	$>10^{-6}$	$>10^{-6}$
$20\text{-epi-}1α,25(OH)_2D_3$	3×10^{-9}	5×10^{-12}
$20\text{-epi-}24a,26a,27a\text{-tri-homo-}1α,25(OH)_2D_3$	1×10^{-9}	1×10^{-12}
$22\text{-oxa-}24a,26a,27a\text{-tri-homo-}1α,25(OH)_2D_3$	3×10^{-9}	1×10^{-10}
$20\text{-epi-}22\text{-oxa-}24a,26a,27a\text{-tri-homo-}1α,25(OH)_2D_3$	3×10^{-9}	5×10^{-11}
Hybrid analogs		
$1β\text{-}20\text{-epi-}24a,26a,27a\text{-tri-homo-}25(OH)_2D_3$	1×10^{-7}	2×10^{-9}
$1β\text{-(hydroxymethyl)-}3α\text{-}20\text{-epi-}22\text{-oxa-}24a,26a,27a\text{-tri-homo-}25\text{-OH-}D_3$	$>10^{-6}$	1×10^{-8}
Analogs with double bond between C16 and C17		
$1α,25(OH)_2\text{-16-ene-}D_3$	1×10^{-9}	1×10^{-10}
$1α,25(OH)_2\text{-16-ene-23-yne-}D_3$	0.8×10^{-9}	6×10^{-10}

[a]ED_{50} (in M), ligand concentration required to reach 50% displacement of $[^3H]1,25(OH)_2D_3$ binding to recombinant human VDR from transfected COS-1 cells.

[b]ED_{50} (in M), effective dose required to produce 50% of maximal transcription activation of a reporter gene containing the osteocalcin VDRE in ROS 17/2.8 cells in serum-free culture medium. The transcriptional activities of $1,25(OH)_2D_3$ and the last two analogs in the table (16-ene analogs) were also examined in cells grown in 10% serum. Under these conditions, the ED_{50} of the three compounds were 5×10^{-10} M, 6×10^{-12} M, and 2×10^{-11} M, respectively.

their transcriptional activity increases manyfold without a change in their affinity for the VDR [56,57].

C. The D-ring

The crystal structure of VDR-1,25(OH)$_2$D$_3$ complex show that the D-ring forms several hydrophobic interactions in the ligand-binding pocket, although these interactions are less critical than those contributed by the A-ring and the side chain [43]. A modification that enhances VDR-mediated transcriptional activity of the hormone is insertion of a double bond between carbons 16 and 17 in the D-ring [58]. Compounds containing this modification, with or without additional chemical modifications in the side chain, have transcriptional activity significantly greater than that of 1,25(OH)$_2$D$_3$ without a significant increase in their affinity for VDR. In addition, this group of analogs exhibits diminished calcium regulating activity. The unique properties of these compounds include lower affinity for vitamin D–binding protein and slower catabolism [59,60], both of which may increase potency (Table I). It is not known what the unsaturation at C16-C17 is doing to the mode of ligand interaction with the VDR, but dot maps suggest that this modification restricts the flexibility of the side chain, and therefore has the potential to force it into contact points that are distinguishable from those used by the side chain of the natural hormone [61].

In conclusion, the A-ring regulates high affinity for VDR, the D-ring controls ligand uptake and metabolism and possibly contributes to the flexibility of the side chain, and the side chain regulates both affinity for VDR and its transactivation (Fig. 1).

III. DIFFERENTIAL ACTIVATION OF THE VDR BY SYNTHETIC ANALOGS

A large amount of information on the structure of the LBDs of various nuclear receptors bound to natural or synthetic ligands has accumulated in the past few years. These studies provide evidence for the structural flexibility of nuclear receptors and a partial explanation for the mechanism of action of agonists, antagonist, and selective receptor modulators. Additional studies, using biochemical, molecular, and cellular biology approaches, provide ample evidence to the many ways by which nuclear receptor actions can be manipulated and refined by synthetic ligands. Interestingly, seven years ago we had little evidence for similar flexibility and heterogeneity in transcriptional activation of the VDR. However, today we are able to distinguish at least three groups of ligands that modulate the VDR

differently from the natural hormone. These include superaognists (such as the 20-epi analogs mentioned above), antagonists, and tissue- or gene-selective agonists. Unfortunately, structural studies provide only partial explanation for the mechanism of action of superagonist, and structural data is not yet available for VDR's LBD bound to antagonists or to tissue-selective agonists. Therefore, the text below will describe primarily biological and biochemical experiments that provide evidence for the differential activation of the VDR by these three groups of compounds.

A. Superagonists

1. DEFINITION AND ASSESSMENT OF SUPERAGONISTS

Superagonists are analogs that are significantly more transcriptionally potent or efficacious than 1,25(OH)$_2$D$_3$. Earlier studies of analogs' actions used primarily ligand-binding assays (to determine affinity for the VDR) and cellular assays (to determine antiproliferative activities) [41]. More recently, the identification and assessment of their activities is accomplished by using common molecular endocrinology methodologies to assess transcriptional activation of genes by steroid hormone receptors. When several DNA binding sites for VDR-RXR complexes in the promoters of vitamin D responsive genes were characterized, the most common tool to assess transcriptional activity induced by analogs became reporter gene assays, in which a fusion gene containing a vitamin D response element attached to a heterologous promoter and a reporter gene is transfected into eukaryotic cells that express the VDR [62–64].

A typical dose-response curve of reporter gene expression in cells treated with the natural hormone shows a gradual induction of reporter gene expression that may reach plateau at 10 nM, with an effective dose for 50% of maximal activity (ED$_{50}$) of 1–5 nM. A superagonist by definition is an analog that induces transcription with an ED$_{50}$ significantly lower than that of the natural hormone, or that induces severalfold greater maximal transcription [Fig. 4 and ref. 21]. The advantages of this assay are that, unlike the growth-inhibitory assay, it directly measures a single VDR-mediated transcriptional activity, it is not subject to other vitamin D–mediated signaling that may take place in cell growth assays, and it is not dependent on vitamin D–mediated effects on RNA stability. It is also faster and more reproducible than the growth inhibitory assays. Its disadvantage is that, in eukaryotic cells (especially mammalian cells), transcriptional activity may still depend on analog uptake (which may vary with differences in binding to DBP in the serum) [65]
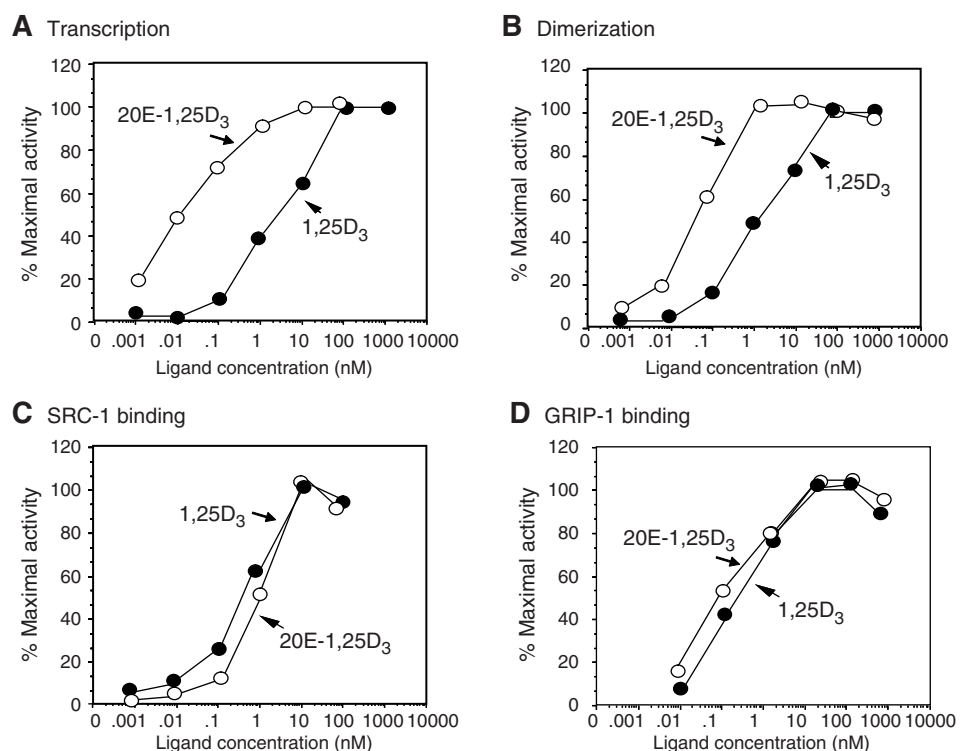
A Transcription

B Dimerization

C SRC-1 binding

D GRIP-1 binding

FIGURE 4   Transcriptional potency of the superagonist 20-epi-1,25(OH)$_2$D$_3$ is correlated with an increase in dimerization potency but not with an increase in binding to the p160 coactivators SRC-1 and GRIP. Transcription (**A**) was assessed by co-transfecting CV-1 cells with the hVDR and a reporter containing the osteocalcin VDRE attached to the minimal thymidine kinase promoter and the growth hormone gene. Dimerization (**B**) binding to SRC (**C**) and binding to GRIP (**D**) were assessed by pull-down assays, using GST-fusion protein to bind the ^{35}S-labeled *in vitro* synthesized hVDR. Bound VDR was eluted, separated by SDS-PAGE, visualized by autoradiography, and quantified by densitometry scanning. These experiments suggest that the dimerization interface rather than the coactivator binding interface is distinct in the VDR-hormone and VDR-20-epi analog complexes [69].

and on the cellular metabolism of the analog [66,67]. For these reasons, it is necessary to confirm differential activation of the VDR by testing analogs in eukaryotic systems that do not contain vitamin D metabolic enzymes (such as yeast) [68], by cell-free transcriptional assays, or by other *in vitro* assays that examine individual ligand-dependent events that are essential for transcriptional activity *in vivo* (binding to DNA or interactions with dimerization partners and with other partners of transcription) (Fig. 4) [21,69].

2. THE 20-NATURAL SUPERAGONISTS

Using these approaches, several analogs have been identified that have greater transcriptional potency than 1,25(OH)$_2$D$_3$. The common feature of these compounds is that they have side-chain modifications but an unmodified A-ring. These analogs' modifications, such as unsaturation at C-23 or fluorine atoms at C-24, limit their availability to 24-hydroxylase [41].

Additional modifications, such as substitution of hydrogen for fluorine atoms at positions 26 and 27 or homologation at these positions, alter access of these compounds to the 26-hydroxylase in the kidney [70]. However, these modifications also have enormous effect on antiproliferative activity and transcriptional response as determined by reporter gene assays in cultured cells. As mentioned above, another modification, unsaturation at positions 16–17 of the D ring, also increases the transcriptional potency of these compounds. This modification alone, or in combination with the aforementioned side-chain modifications, has been shown to slow down 24-hydroxylation and cause cellular accumulation of active catabolic intermediates [71]. Therefore, it appears that almost all of the increases in the potencies of compounds from this group are due to altered pharmacokinetics, including a decrease in binding to DBP that *in vitro* causes the cells to take up the analogs faster than 1,25(OH)$_2$D$_3$ [65]. However, several studies

have provided *in vitro* evidence for a true change in their ability to induce receptor-mediated activity. First, Uskokovic *et al.* have shown that the analogs containing the 16-ene modification plus hexafluorine substitutions at the 26 and 27 positions have a twofold greater affinity for the VDR than the natural hormone [71], although this affinity is not proportional to the increase in transcriptional potency (100- to 1000-fold) [72]. Another study has shown that several fluorinated compounds increase the affinity of the VDR-analog complex for its dimerization partner RXR or increase the affinity of the VDR-RXR complex for DNA [72]. Again, these changes are not proportional to the observed increases in transcriptional activities of these compounds in culture, suggesting that these modifications may affect additional parameters of transcriptional activation of the VDR.

3. THE 20-EPI SUPERAGONISTS

The best-characterized modulators of the VDR are the 20-epi analogs, including 20-epi-1α,25(OH)$_2$D$_3$ (MC-1288) and 20-epi-22-oxa-24a,26a,27a-tri-homo-1α,25(OH)$_2$D$_3$ (KH-1060) (Table I) [55]. These compounds, synthesized by Leo Pharmaceuticals, share one distinct modification, a stereochemical change at carbon 20. Dot maps revealed that the energy-minimized side chain conformers of these analogs have mostly northwest orientations, instead of the northeast orientations of side chains of the natural hormone and 20-natural analogs [73]. Without chemical modification of the 20-epi side chain, the analog 20-epi 1,25(OH)$_2$D$_3$ (MC-1288) has a hundredfold greater growth-inhibitory and transcriptional activities than 1,25D$_3$ does. The addition of chemical modifications to the 20-epi side chains in the analogs KH-1060 and MC-1301 (20-epi-24a,26a,27a-tri-homo-1α,25(OH)$_2$D$_3$) increased their activities up to 3,000-fold compared with that of 1,25D$_3$ [21,55,74]. These phenomenal improvements in activities are not associated with any increase in affinity for the VDR [21,55]. These compounds act directly by altering VDR actions, as they induce reporter gene expression more effectively than 1,25D$_3$ in many cell lines and through several types of vitamin D–response elements [21]. These results occurred in both the presence and the absence of DBP, thus reducing the probability that altered DBP-modulated delivery of these ligands contributes to their potency in culture. It has been shown that 24-hydroxylase does not effectively catabolize several 20-epi analogs [67,75]. However, that they are still significantly more potent than 1,25(OH)$_2$D$_3$ in cells lacking 24-hydroxylase activity (ROS 17/2.8) [21] excludes differential catabolism as a primary explanation for their potency. The most compelling evidence for the

molecular basis of the enhanced activity of these compounds came from a series of studies demonstrating, in a yeast two-hybrid system, that the 20-epi analog KH-1060 induces dimerization more effectively than 1,25(OH)$_2$D$_3$ does [68]. More importantly, by using cell-free assay systems, it was shown that binding of 20-epi analogs to the VDR induces a conformation distinct from that induced by the natural hormone [21]. This distinct conformation is associated with the enhanced ability of VDR-20-epi analog complexes to dimerize with RXR [69,76]; to bind to specific DNA sequences (VDREs) [21]; to interact with a key component of the transcription apparatus, DRIP 205; and to induce transcription in cell-free systems [77] (Fig. 4).

These biochemical findings strongly suggest that the contact points of 20-epi analogs with the VDR are different from those used by the natural hormone and that these differences cause conformational changes that affect the properties or availability of VDR binding domains for transcription partners (Fig. 4). Biochemical evidence that the modes of interaction of the natural hormone and the analogs are different came from studies of site-directed mutagenesis of the VDR and from comparing the effects of mutations on binding of the hormone and the 20-epi analogs [78]. These experiments showed that the ability of the 20-epi analogs to interact with a VDR that lacked contact sites essential for binding of 1,25D$_3$ is not impaired. The difference in binding requirement appears to involve residues in the C-terminal region of the VDR, including the AF-2 core/helix 12. This domain, which is essential for coordinating the interaction of coactivators with the VDR, also has contact points for the side chain of 1,25D$_3$ but appears to be less important for the binding of the 20-epi analogs to VDR. Studies demonstrating that the half-lives of the VDR-20-epi analog complexes were significantly greater than the half-lives of VDR complexes with their 20-natural counterparts suggested that the 20-epi analogs may be buried more deeply in the binding pocket than the 20-natural compounds are [78]. Interestingly, structural analysis of the VDR-20-epi analog complexes by X-ray crystallography did not support the biochemical data, because it did not show that the 20-epi analogs use contact points different from those used by 1,25D$_3$, unless the analogs had chemical modifications in their side chains. Furthermore, the structural studies did not provide evidence for a significant change in the functional surface of the VDR-20-epi analog complexes that would explain the modified interactions of these complexes with dimerization partners and with the bridging factor DRIP 205 *in vitro* [79]. An explanation for this discrepancy could be that 20-epi analogs shift their position in the ligand-binding pocket when the VDR is associated with transcription
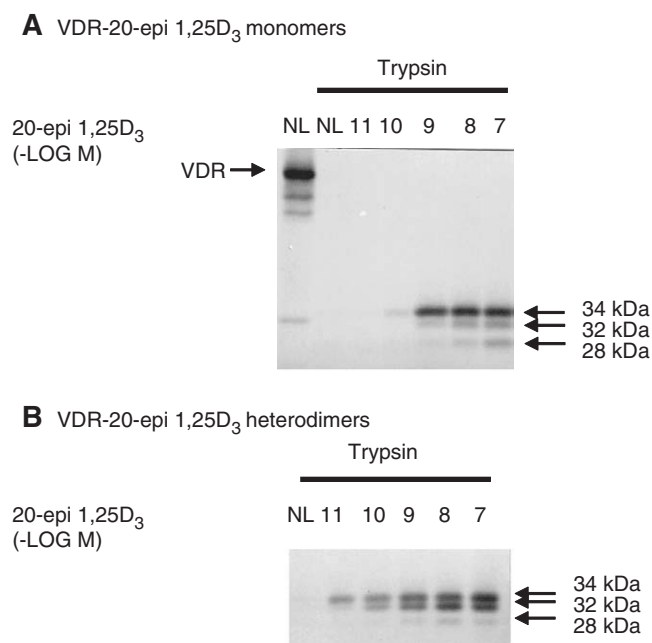
A VDR-20-epi 1,25D$_3$ monomers

Trypsin

20-epi 1,25D$_3$
(-LOG M)   NL NL 11 10 9   8   7

VDR →

34 kDa
32 kDa
28 kDa

B VDR-20-epi 1,25D$_3$ heterodimers

Trypsin

20-epi 1,25D$_3$
(-LOG M)   NL 11 10 9   8   7

34 kDa
32 kDa
28 kDa

FIGURE 5   The effect of VDR interaction with transcriptional partners on the mode of analog binding. (**A**) *In vitro* translated VDR was incubated with 20-epi analog (20-epi-1,25(OH)$_2$D$_3$) and then subjected to trypsin digestion. (**B**) *In vitro* translated VDR was incubated with the 20-epi analog, with GST-RXR and gluatathione-Sepharose beads. VDR-RXR complexes were separated from the unbound VDR and then subjected to trypsin digestion. Note the differences in the conformation of VDR-analog complexes with RXR and VDR-analog monomers. Also, note the 100-fold increase in the ability of the analog to stabilize the conformation of the heterodimerized VDR [69].

partners [83] (Fig. 5), and these changes are not reflected in the monomer structure that was analyzed by X-ray crystallography. Additional explanation could be that VDR bound to 20-epi analogs in the cells undergoes modifications that enhance its abilities to bind DNA and partners of transcription, and these modifications do not occur in the cell-free systems [80,81].

B. Low-calcemic Analogs/Selective Agonists

1. ASSESSMENT OF NONCALCEMIC SELECTIVE AGONISTS

Surprisingly, a major misconception exists in the vitamin D field because analogs have been defined as noncalcemic or low-calcemic if they have significant receptor binding activity *in vitro* and growth-inhibitory activity in culture but when administered to animals do not induce hypercalcemia/hypercalciurea at the concentration range at which 1,25(OH)$_2$D$_3$ does [41]. However, low calcemic activity *in vivo* may simply be due to pharmacokinetic properties such as rapid clearance

rate and short terminal half-life, which would lead to very poor overall biological activities [70]. Therefore, a more accurate definition for low-calcemic analog is a compound that has a wider safety window than 1,25(OH)$_2$D$_3$ to induce a biological response without inducing hypercalcemia [82]. We define analogs that have these desirable qualities *in vivo* as "selective" agonists because, by definition, the ability to induce biological response without changing calcium homeostasis requires that the analog has a preference for a given target tissue (e.g., tumor cells, the parathyroid gland, and skin) over calcium-regulating organs such as intestine, kidney, and bone. In this review, we will discuss only those low-calcemic selective agonists that have shown evidence of regulating VDR functions differently from 1,25(OH)$_2$D$_3$ in culture and in cell-free systems.

2. MECHANISM OF ACTION OF SELECTIVE AGONISTS

The best-characterized analogs that can be defined as low-calcemic but biologically active at a reasonable concentration range *in vivo* and as selective modulators of the VDR *in vitro* are three structurally unrelated compounds (Fig. 6). One is Leo Pharmaceuticals' EB-1089, a side chain-modified analog (22,24-diene-26,27-bishomo-1,25-dihydroxyvitamin D$_3$) that inhibits tumor growth *in vivo* with half the calcemic activity of 1,25(OH)$_2$D$_3$. Another of these analogs is Chugai's OCT (22-oxa-1,25-dihydroxyvitamin D$_3$), also a side chain modified analog that inhibits parathyroid hormone secretion without inducing hypercalcemia. The third analog is Roche's Ro-26-9228 (1αF-16-ene-20-epi-23-ene-26,27-bishomo-25-hydroxyvitamin D$_3$), a hybrid analog that restores bone loss without inducing hypercalcemia at a wide concentration range.

Examination of the mechanism of action of these apparent selective agonists has raised three questions: (1) Is there evidence that their mode of interaction with and transcription activation of the VDR is significantly different from that of the natural hormone? (2) Is there evidence that gene expression in cells or tissues that regulate calcium homeostasis is modulated differently by the natural hormone and by these analogs? (3) Is there compelling evidence that the analog has tissue or gene preference different from that of 1,25(OH)$_2$D$_3$? The compounds described below each have several of these features. However, additional studies are required to further evaluate and substantiate the mechanisms for their apparent selective actions.

Leo Pharmaceutical developed EB-1089 primarily for use in chemotherapy of malignancies, with a focus on breast cancer [83–85]. *In vivo*, EB-1089 has a reasonable safety window for inhibiting tumor growth without hypercalcemia, but how that occurs is not
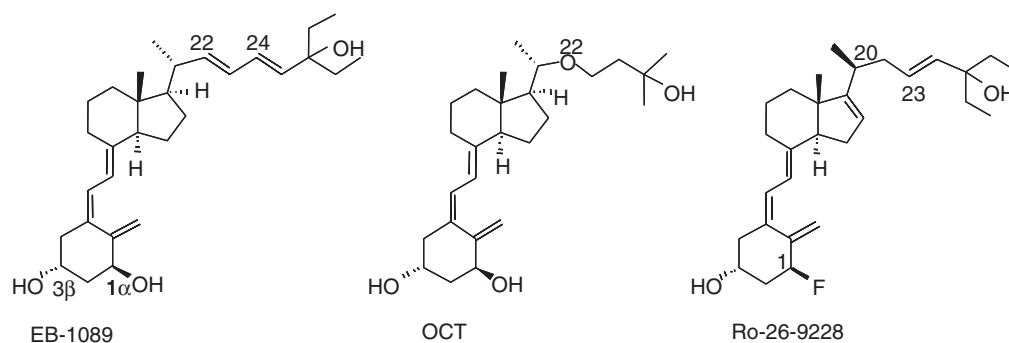
FIGURE 6   Structural formulas of selective agonists. EB-1089 was synthesized by Leo Pharmaceuticals; OCT was synthesized by Chugai, and Ro-26-9228 was synthesized by Roche Bioscience.

known [84,86]. There have been few attempts to distinguish EB-1089's mechanism of VDR activation from VDR-mediated actions of $1,25(OH)_2D_3$. *In vitro* assays demonstrated that the electrophoretic mobilities of VDR-EB-1089-DNA complexes and VDR-$1,25(OH)_2D_3$-DNA complexes are different, suggesting that the conformations of the complexes or their compositions are different [87]. Another study showed that EB-1089-VDR complexes have an apparent binding preference for IP9 (inverted repeat with nine intervening nucleotides) VDRE instead of the classic DR3 (direct repeats with three intervening nucleotides) VDREs, whereas $1,25(OH)_2D_3$-VDR complexes do not. Cell culture studies also revealed that EB-1089, but not $1,25(OH)_2D_3$, has some preference for inducing transcription through the IP9 VDRE of a transfected reporter gene [87,88]. Because the response elements used were synthetic and not present in natural genes, the relevance of these findings is not clear, except that they showed differences in the molecular properties of EB-1089-VDR and $1,25(OH)_2D_3$-VDR complexes. Unfortunately, these studies were not extended to test cause and effect relationship between these phenomena and the pharmacological actions of EB-1089 *in vitro* and *in vivo*.

The results of cell-culture and *in vivo* studies to determine the mechanism for the low calcemic activity of EB-1089 have been somewhat equivocal and provide only a partial explanation for its selective activities. Cell culture studies have shown that EB-1089 is as good or better than $1,25(OH)_2D_3$ at inducing bone resorption [89]. *In vivo* studies demonstrated that EB-1089 is somewhat less effective than $1,25(OH)_2D_3$ at inducing 24-hydroxylase and calbindin D9K mRNAs in the duodenum [90]. On the other hand, EB-1089 and $1,25(OH)_2D_3$ have similar abilities to induce these genes in the kidney. These results suggest that EB-1089 has different preference for the duodenum than

$1,25(OH)_2D_3$ and that the lower calcemic activity of EB-1089 may be associated with a lower ability to induce gene expression at a site that regulates calcium absorption [90]. It may also suggest that EB-1089 has a different tissue preference *in vivo*. In conclusion, these *in vitro* and cell culture studies provide some evidence that EB-1089 is a selective modulator of the VDR, and that its selectivity may be either at the level of target genes or target tissues, but additional studies *in vivo* and *in vitro* are necessary to substantiate these findings.

Another side chain modified analog, OCT, has been shown to be potent in animals without inducing hypercalcemia [41]. The pharmacokinetic properties of this analog are significantly different from those of $1,25(OH)_2D_3$. First and foremost, OCT binds very poorly to DBP [91], which may contribute to its short half-life in animals. However, there is strong evidence that this analog has a selective action *in vivo*, as it induces only brief intestinal calcium absorption but prolonged inhibition of parathyroid hormone secretion. The brief intestinal calcium absorption correlates with transient induction of calbindin D9K, a vitamin D receptor-modulated gene. The brief induction of calbindin D9K by OCT is significantly different from the longer $1,25(OH)_2D_3$-dependent induction of the gene [91–93]. Two interesting features about these differences in gene expression between the two compounds are that the analog is retained in the intestine longer and that its maximal binding to the intestinal VDR is higher than that of $1,25(OH)_2D_3$. These findings suggest that the brief period of gene expression is not due to a short half-life of the intestinal VDR-OCT complexes, but perhaps to transcriptional activation events downstream of the formation of VDR-ligand complexes.

That OCT is indeed a selective agonist has been supported by *in vitro* assays that showed differences in

recruitment of transcriptional coactivators to VDR-OCT and VDR-1,25(OH)$_2$D$_3$ complexes [94]. However, the assays did not provide direct proof that the coactivator selectivity of VDR-OCT complexes *in vitro* leads to cell-type selective or gene-selective actions *in vivo*.

Another example of an analog with convincing tissue- and cell-selective properties is the Roche compound Ro-26-9228 [82]. Biochemically it is a "hybrid" analog because it contains modifications in both the A-ring and the side chain. Its affinity for VDR is 8- to 10-fold lower than the affinity of the natural hormone, as would be expected from replacement of the 1α-OH group with a fluorine atom. However, examination of its transcriptional activities in cell culture showed that Ro-26-9228 is equipotent to the natural hormone in osteoblasts, whereas in intestinal cells it is 60 times less potent (Fig. 7 and [82]). Further studies of the VDR from the two cell types has revealed that when it binds the analog in intestinal cells, it does not acquire the ability to interact with dimerization partners and transcription coactivators, whereas when the analog binds the VDR from osteoblasts or synthetic VDR *in vitro*, it does have these abilities. In contrast, the natural hormone 1,25(OH)$_2$D$_3$ has these abilities in both cell types [95]. This apparent cell selectivity *in vitro* mimics the tissue preference *in vivo*: the administration of Ro-26-9228 to rats induces VDR–dependent gene expression in the bone but not in the duodenum (Fig. 8 and [82]). These properties of the analog *in vivo* are associated with prevention of bone loss in osteopenic rats without induction of hypercalcemia over a very wide concentration range. This suggests that poor recognition of the analog by the VDR in the duodenum spares the animals from hypercalcemia induced by enhanced calcium absorption, whereas the analog's preference for bone (probably for osteoblasts) promotes bone-remodeling activities that lead to a net bone gain [82].

C. Antagonists

1. Assessment of Antagonists

Antagonists are receptor-binding compounds that inhibit the actions of the respective natural hormone and, on their own, are unable to elicit a transcriptional response through the receptor. Therapeutically, these compounds are exceedingly valuable, as they are used to prevent growth of hormone-responsive malignancies (estrogen antagonists) [96–98] and to regulate reproductive processes (progesterone antagonists) [99]. Only a few of these compounds are pure antagonists, but in many cases they may act as antagonists on certain target genes or in certain tissues and as moderate

A Transcription in Caco-2 cells

ED$_{50}$
1,25D$_3$ = 2 nM
Ro-26-9228 = 120 nM

B Transcription in MG-63 cells

ED$_{50}$
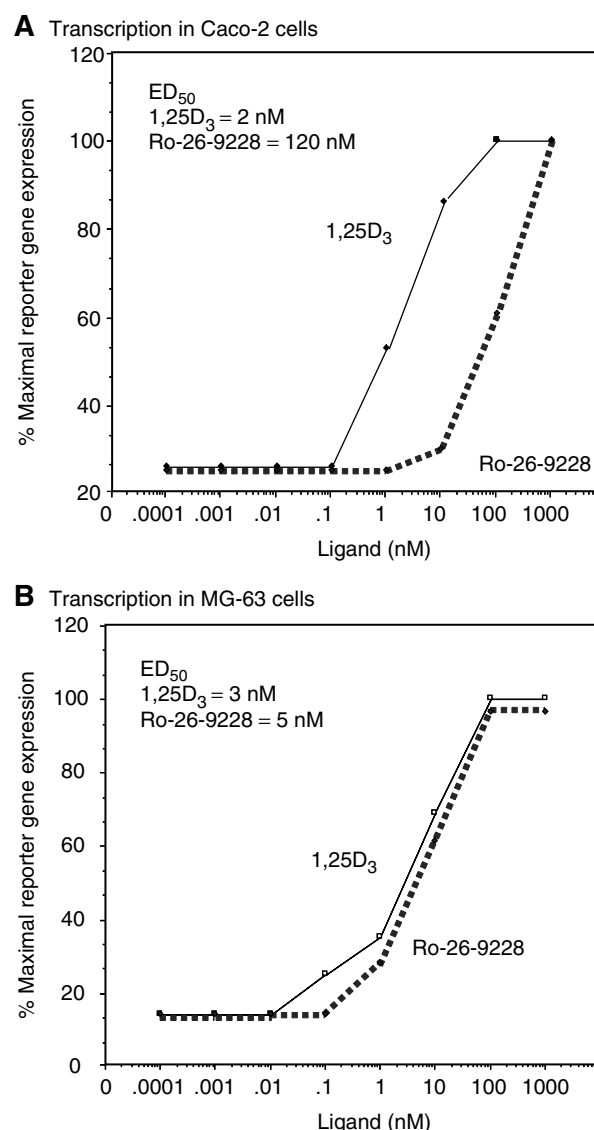1,25D$_3$ = 3 nM
Ro-26-9228 = 5 nM

Figure 7 A selective modulation of VDR-mediated transcription in cultured cells. Human colon carcinoma cells (Caco-2) or human osteosarcoma cells, MG-63 were transfected with a reporter gene containing a minimal thymidine kinase promoter and a vitamin D response element. The cells were treated with the indicated doses of 1,25(OH)$_2$D$_3$ or the analog Ro-26-9228, and reporter gene expression was assessed 48 h later. Note that the analog is equipotent to 1,25(OH)$_2$D$_3$ in the osteoblast-like cells but is 60 times less potent than 1,25(OH)$_2$D$_3$ in the intestinal-like cells [82].

agonists in others. These properties opened numerous therapeutic possibilities that are best represented by the synthetic estrogen receptor-binding ligands termed selective estrogen receptor modulators (SERMs) that include tamoxifen and raloxifene [96,97]. Their antagonistic properties are used to inhibit the growth of estrogen-dependent breast cancer cells, while their agonist activities are used to maintain bone integrity in
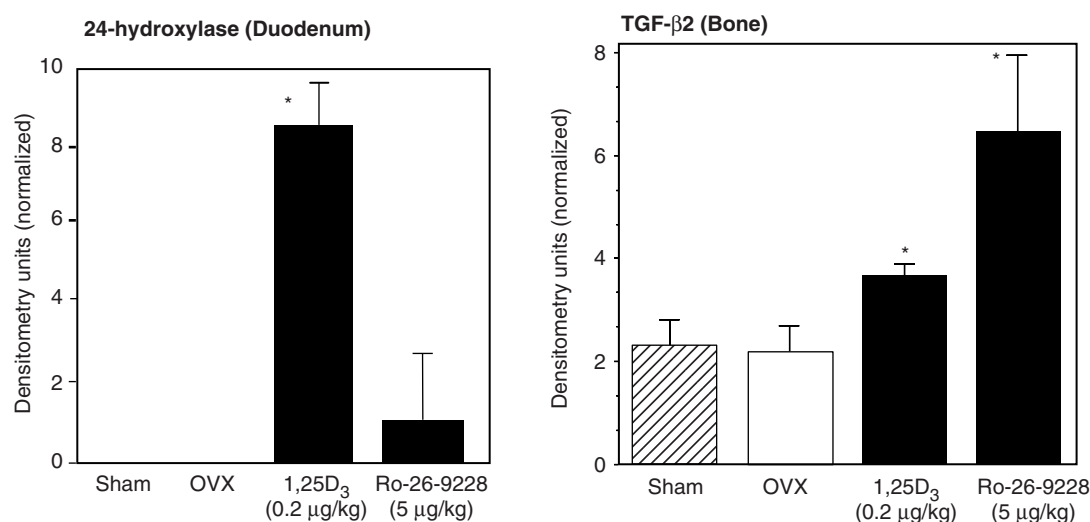
FIGURE 8   A selective modulation of gene expression in target tissues for vitamin D action by the analog Ro-26-9228, *in vivo*. Female rats were either sham-operated or ovariectomized for three weeks and then given vehicle, or the indicated dose of the vitamin D compound. Total RNA was isolated from the duodenum and the tibia of these animals 7 h after treatment. The mRNAs were quantified by northern blots (24-hydroxylase) or by semiquantitative RT-PCR (TGF-β2). Note that Ro-26-9228 did not induce a significant gene expression in the duodenum whereas it did so in the bone. In contrast $1,25(OH)_2D_3$ induced expression of these genes in both tissues [82].

postmenopausal women. At the molecular level, binding of antagonists to their respective receptors induces a conformational change that is not permissive for recruitment of coactivators. In fact, antagonist binding prevents coactivator binding *in vitro* by causing a conformational change in the position of the C-terminal AF-2 core [46]. The cellular conditions that render these complexes transcriptionally active are debated but include changing ratios of coactivators and corepressors, and interaction of another transcription activation domain (the N-terminal AF-1) with the transcription apparatus under conditions that are not permissive for this interaction through the AF-2 domain of the antagonist-bound receptor (Fig. 3 and [98,99]).

Numerous vitamin D analogs have been synthesized, but until five years ago none exhibited clear antagonistic activities. One reason could be the remarkable structural flexibility of vitamin D derivatives (unlike the relatively rigid four-ring steroid hormones) [100]. This flexibility may also allow these ligands to adapt their conformations to the binding pocket of the VDR despite many structural modifications, and therefore vitamin D analogs may be less likely to disrupt formation of a functional VDR surface. Another possibility is that, for practical reasons, vitamin D antagonists are not as obviously useful clinically as selective agonists might be, and therefore have not been synthesized strategically or have not been investigated. Despite these limitations, there are two groups of vitamin D analogs with significant

antagonistic activities. The best representative of the first group is the 26,23-lactone TEI 9647, synthesized at the Teijin Institute for Biomedical Research. The other group of antagonists is the carboxylic esters, the most potent representative of which is ZK 159222, synthesized at Schering AG (Fig. 9).

2. THE LACTONES

The lactone analog TEI 9647 (23S-25-dehydro-1α-hydroxyvitamin D_3-26,23-lactone) has an affinity for VDR which is one-tenth that of $1,25(OH)_2D_3$ [101]. Its mode of interaction with the VDR is different from that of $1,25(OH)_2D_3$, because the analog stabilizes a conformation that is significantly different from that stabilized by $1,25(OH)_2D_3$ [102]. This distinct
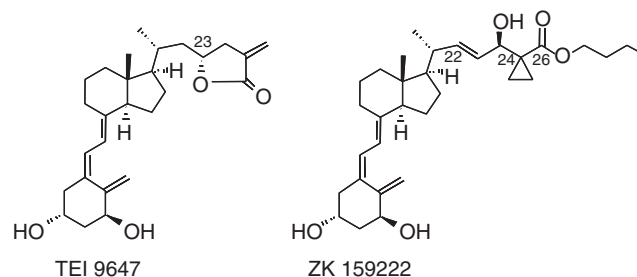
Antagonists

FIGURE 9   Structural formulas of antagonists: TEI 9647 was synthesized by Teijin, and ZK 159222 was synthesized by Schering.

conformation is evident in the VDR monomer and in the VDR complexes with RXR and with DNA [103,104]. It is possible that the antagonist activities are due to disruption of the conformation of helix 12 by the bulky lactone group at the side chain of this compound. This disruption causes the inactivation of the AF-2 domain (Fig. 3) and a loss of transcriptional activity of the antagonist-bound VDR. However, X-ray crystallography of the VDR bound to this ligand confirms that hypothesis has not yet been performed. Despite the lack of structural information, this analog can effectively inhibit $1,25(OH)_2D_3$-mediated transcriptional activity in COS-7 cells cotransfected with the human VDR and a reporter gene containing the 24-hydroxylase VDREs [103]. It also inhibits $1,25(OH)_2D_3$-mediated transcription in Saso-2 cells transfected with the same reporter but with the endogenous VDR, and in MCF-7 cells transfected with a reporter gene containing a single DR3-type VDRE [103]. The antagonistic activity of TEI 9647 seems to involve two LBD-mediated actions: the analog appears to inhibit $1,25(OH)_2D_3$-induced VDR-RXR dimerization and to inhibit interaction of VDR with the p160 coactivator SRC-1 [103,104].

Using HL60 cells as a model for the growth inhibitory and differentiating actions of $1,25(OH)_2D_3$, cellular responses that are largely considered to be VDR-mediated, it was shown that TEI 9647 did not induce any of the differentiation markers induced by $1,25(OH)_2D_3$ and prevented all aspects of the growth-inhibitory and differentiating actions of $1,25(OH)_2D_3$ [101]. These results underscore the notion that growth inhibition and differentiation induced by $1,25(OH)_2D_3$ are indeed mediated through the nuclear VDR, although some aspects of these cellular processes are thought to be mediated through a distinct membrane receptor for $1,25D_3$ (see Chapter 23).

TEI 9647 was examined for antagonistic and agonistic activities in normocalcemic rats and in vitamin D–and calcium-depleted rats [105]. TEI 9647 has a moderate ability to inhibit the increase in serum calcium in normocalcemic rats injected with pharmacological amounts of $1,25(OH)_2D_3$, but this analog does not inhibit the normal physiological activities of $1,25(OH)_2D_3$. In vitamin D–deficient and calcium-deficient rats, TEI 9647 acts as a poor agonist of intestinal calcium absorption and a somewhat better agonist of bone resorption, and it very effectively inhibits parathyroid hormone secretion. Interestingly, when given together with $1,25(OH)_2D_3$ to the vitamin D–deficient rats, TEI 9647 can inhibit the calcium-absorbing and bone-resorbing activities of $1,25(OH)_2D_3$ as well as the $1,25(OH)_2D_3$-mediated inhibition of parathyroid hormone secretion [105].

These results suggest that under normal physiological conditions *in vivo*, TEI 9647 is a poor antagonist.

In contrast, *in vitro* it can antagonize a wide range of $1,25(OH)_2D_3$-mediated VDR actions without a target gene or target tissue preferences. Therefore, it is not likely that TEI 9647 has mixed agonist-antagonist activities that would make it useful in a clinical setting. However, all of the *in vitro* experiments on TEI 9647 were performed with human cells or recombinant human VDR, and the *in vivo* experiments were performed with rats. One might speculate that the analog might not be an effective antagonist of the rat VDR as it is of the human VDR, and therefore its actions in the rat may differ from those in humans.

3. THE 26-CARBOXYLIC ESTERS

The other group of antagonists is the carboxylic esters ZK 159222 and ZK 168281 [106]. These compounds appear to act as typical antagonists of nuclear receptors *in vitro* and in cultured cells, and in very high doses they act as poor agonists. In the presence of $1,25D_3$, they inhibit VDR-mediated transcriptional activities. *In vitro* they induce a VDR conformation different from that induced by $1,25(OH)_2D_3$, a clear indication of a difference in the mode of interaction with the receptor. These analogs' binding to VDR does not prevent dimerization and binding to DNA (as with the lactone compounds), but they do not induce interaction with transcription coactivators of the p160 family and they partially inhibit the $1,25(OH)_2D_3$-mediated interaction of VDR with these factors [106,107]. These results suggest that the step in VDR activation that is disrupted by the 26-carboxylic esters is the induction of a VDR conformation that allows interaction with the coactivators. Since this is an AF-2-dependent function, it suggests that the long side chain of these analogs disrupts the agonist conformation of the helix 12/AF2 core in the VDR [108]. Without X-ray crystallography data, however, it is not possible to determine whether the AF-2 core in these VDR-analog complexes assumes an antagonist conformation such as that seen in the AF-2 core in estrogen receptor-tamoxifen complexes (e.g., the coactivators binding site is masked) or simply is not able to properly interact with coactivators because its conformation is similar to that of the AF-2 core in the unoccupied receptor (Fig. 3). So far, tissue- or gene-specific activities of these antagonists have not been identified, either in culture or in animal studies.

IV. CLINICAL SIGNIFICANCE FOR SELECTIVE MODULATION OF THE VDR BY VITAMIN D ANALOGS

Vitamin D metabolites (Calderol of Organon, Rocaltrol of Hoffmann-LaRoche) and analogs that are

in fact prehormones of $1,25(OH)_2D_3$ (One-alpha of Leo Pharmaceuticals, Hectoral of Bone Care International) have been used for years for treatment of senile osteoporosis, postmenopausal osteoporosis, secondary hyperparathyroidism, and the skin disease psoriasis [109–116]. These usages suggest that new analogs of vitamin D will be developed and used first and foremost for treatment of these conditions. For instance, the relatively new analog Dovonex (synthesized at Leo Pharmaceuticals) has been used in the past few years for treatment of psoriasis [117–119] and Zemplar (19-nor-$1,25(OH)_2D_3$, Abbott Laboratories) and maxacalcitol (OCT, synthesized at Chugai) have recently been approved for use in secondary hyperparathyroidism [120–122]. Of these compounds, however, only OCT has been established as a selective modulator of the VDR. Other interesting analogs are in clinical trials for osteoporosis, including ED-71 of Chugai [123,124] and Roche's selective modulator Ro-26-9228. Both analogs have been carried to phase II clinical trials in postmenopausal osteoporosis and appear to be well tolerated and effective [82]. The analog Ro-26-9228 (also named BXL628) is currently being tested in clinical trials for treatment of benign prostate hyperplasia (BPH), and it will be used in clinical trials for post-transplantation immunosuppression through the next three years (Dr. L. Adorini, personal communication, and unpublished results). Because the side effects common to drugs used for treatment of these two conditions include bone loss, this bone-protecting vitamin D analog may have a dual beneficial effect for BPH and transplantation patients.

Until recently, there did not seem to be a specific clinical use for vitamin D antagonists. However, recent studies on Paget's disease have suggested a specific increase in osteoclasts' sensitivity to the differentiating action of $1,25(OH)_2D_3$ as the principal underlying mechanism for abnormal bone formation in patients with this disease. These findings suggest that potent vitamin D antagonists might be useful drugs to inhibit the abnormal activation of osteoclasts in this disease [125,126].

Another clinical condition that has not yet been explored is the osteolytic form of metastatic bone disease. This form of bone metastases is common in multiple myeloma and renal carcinoma, and is associated with massive activation of osteoclast actions. Osteoclast activation in osteolytic metastatic bone disease may be coupled to osteoblasts' production of cytokines that promote osteoclast differentiation and function (primarily RANKL) [127]. Therefore, it remains to be examined whether $1,25(OH)_2D_3$-regulated cytokine production by osteoblasts can be blocked in this form of metastatic bone disease by vitamin D antagonists, without adverse effects on calcium homeostasis and bone turnover.

One of the most attractive pharmacological features of vitamin D analogs is their ability to inhibit malignant cell growth *in vitro* and in animal models. These effects depend on the ability of these compounds to induce cell differentiation or apoptosis and to inhibit angiogenesis *in vivo* [85,128,129]. One of the compounds most thoroughly studied in that respect is the selective modulator EB-1089 (secocalcitol of Leo Pharmaceuticals). In preclinical studies, it inhibited various malignancies including breast, colon, and prostate cancers [84,86,130,131]. EB-1089 even has a significant effect on progression of breast cancer cells into bone in nude mice [132]. In culture and *in vivo*, EB-1089 appears to have significant differentiating and apoptotic effects and its inhibition of breast cancer cells metastasis into bone suggests that it is also effective on angiogenesis, an important parameter of tumor progression. Interestingly, EB-1089 has been successful in clinical trials of advanced liver cancer and is also being tested in advanced pancreatic and breast cancer [133,134].

Although EB-1089 is efficacious in animal models of malignancies and also has a significant effect on several human malignancies, it still exhibits significant calcemic. Consequently activity, its therapeutic window is somewhat limited [130,133]. Therefore, the future development of additional low-calcemic analogs that are potent inhibitors of tumor cell growth and have a wider therapeutic window will be necessary to further establish vitamin D analogs in cancer therapy.

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Development of New Vitamin D Analogs

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I. INTRODUCTION

The involvement of LEO Pharma in the synthesis and evaluation of new vitamin D analogs and metabolites dates back to the early 1970s, with the development of 1α-hydroxycholecalciferol [$1\alpha(OH)D_3$] for the treatment of renal osteodystrophy and hyperparathyroidism. Our interest was further stimulated in the early 1980s with the appearance of reports describing receptors for $1\alpha,25$-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] in nonclassic target tissues and the demonstration of the role of $1,25(OH)_2D_3$ in regulating growth and differentiation of various cancer cell lines, *in vitro* and *in vivo* [1,2]. At the same time, a number of reports suggested that $1,25(OH)_2D_3$ might also influence various functions of activated lymphocytes and thereby play a role as a physiological regulator of the immune system [3,4].

These findings suggested new therapeutic possibilities for $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$, especially in neoplastic and immune-mediated diseases. In 1983, LEO took steps to initiate clinical trials with $1\alpha(OH)D_3$ in leukemia and non-Hodgkin's lymphomas [5,6]. The therapeutic usefulness of $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$ was, however, likely to be limited by their potent effects on calcium metabolism, leading to side effects such as hypercalcemia and soft tissue calcifications. It was therefore decided to try to develop new analogs with a more favorable therapeutic profile.

In 1985, the preliminary testing of a small series of new synthetic analogs led to the discovery of a promising candidate, MC903, later named *calcipotriol*. At the same time, clinical observations suggested that $1\alpha(OH)D_3$ and $1,25(OH)_2D_3$ might exert antipsoriatic effects [7,8]. It was therefore decided to test calcipotriol in patients with psoriasis and to further expand our engagement in vitamin D chemistry. This chapter reviews our main efforts and achievements in this field since the mid-1980s.

II. STRATEGY FOR DEVELOPMENT OF NEW VITAMIN D ANALOGS

A. Basic Screening Strategy

At the start of the program for synthesis and evaluation of new vitamin D analogs, the primary aim was to identify compounds that were potent regulators of cell proliferation and differentiation, but which had a reduced ability to exert the classic effects of $1,25(OH)_2D_3$ on calcium homeostasis. Structure-activity relationships of a relatively large number of analogs (more than 1000) have been studied in detail over the last 15 years.

Initially, the effects of all newly synthesized vitamin D analogs were tested in the human histiocytic lymphoma cell line U 937, which expresses the vitamin D receptor (VDR) and responds to $1,25(OH)_2D_3$ with a decrease in proliferation and induction of differentiation along the monocyte-macrophage pathway [9,10]. The analogs were tested in the cell culture system for 4 days, at concentrations ranging from 10^{-12} to 10^{-7}M, and their effects were compared to those of $1,25(OH)_2D_3$. More recently, the U 937 cell cultures were supplemented with other cell types, notably mammary cancer cells (MCF-7, human breast carcinoma cell line) and skin cells (HaCaT, human keratinocyte cell line).

The binding of the vitamin D analogs to the VDR was assessed by displacement of bound ^3H-$1,25(OH)_2D_3$ from receptor protein obtained from the intestinal epithelium of rachitic chickens [11]. As the binding affinity of many analogs did not show a direct correlation

with their biological activities (see also Section IV.A), the VDR binding studies were extended to include studies of the transcriptional activity of the analogs, using the vitamin D responsive element in the promoter region of the osteocalcin gene.

To assess the effects of new vitamin D analogs on calcium metabolism, an *in vivo* model was chosen [10]. The analogs were administered orally to rats, daily for 7 days. Urine was collected daily, and blood was collected by cardiac puncture at the end of the experiment. Metaphyseal bone was prepared from tibiae. Calcium levels were determined in urine and serum samples, and the calcium content in bone was assessed after ashing. To assure detection of even small differences in potency between various analogs, the rats were given a vitamin D–replete diet with a high calcium content (1%), in contrast to many of the older studies in rats and chickens given low calcium and/or low vitamin D diets [12,13].

As a follow-up to the screening system described above, the metabolic stability (serum half-life) of selected analogs was initially tested *in vivo* after intravenous administration to rats [14]. At a later stage, an *in vitro* test using the rat liver post-mitochondrial fraction (S9) was introduced. The analogs were tested at a single concentration, and the percentage of intact analog after 1 hour of incubation was assessed. The analogs were classified as unstable (<10% intact analog), medium stable (10–35% intact analog), and stable (>35% intact analog). Analogs with a low metabolic stability were considered as candidates for topical use, and analogs with a high metabolic stability as candidates for systemic use.

B. Synthesis Strategy

From the beginning of the project it was decided to concentrate our efforts on the synthesis of analogs of $1,25(OH)_2D_3$ (**I**) in which the C-17 side chain was modified while the seco-steroid ring system was kept intact. This decision was partly dictated by the fact that this part of the molecule is more easily accessible to chemical manipulation than the ring system, but it was also decisive that the side chain is known to play a crucial role in the binding of $1,25(OH)_2D_3$ to its receptor [15]. It is beyond the scope of this chapter to detail the synthesis of the more than 1000 analogs that have been made in the LEO laboratories, but some general pathways are outlined in the following sections.

Our starting material has been the readily available ergocalciferol (**II**) (Scheme 1), which, according to a method originally devised by Hesse [16,17], can be converted to a 1α-hydroxylated *trans* vitamin derivative


(**I**)
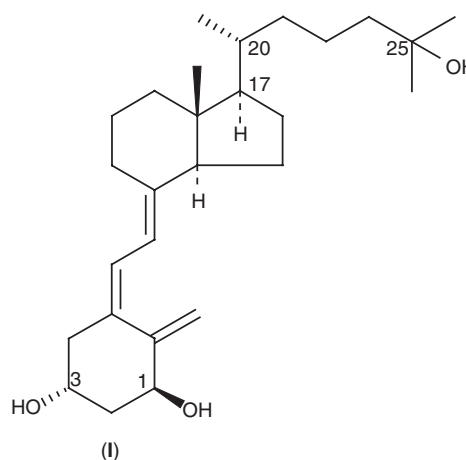
(**IIa**), conveniently isolated as the pure, crystalline, bis-silyl ether (**III**) [18]. After protection of the conjugated triene system as the sulfur dioxide adduct (**IV**), the side chain can be cleaved selectively by ozonolysis with formation of the aldehyde (**V**). By heating (**V**) in the presence of $NaHCO_3$, SO_2 is expelled, and the key intermediate (**VI**) can be isolated as a crystalline compound [18]. Compound (**VI**) has been a cornerstone in our synthetic work.

By means of the Wittig reaction, new side chains could be introduced. Scheme 2 illustrates the synthesis of the anticancer drug EB 1089 (**IX**) (seocalcitol, see Table II), which contains two conjugated double bonds in the side chain [19]. The product of the Wittig reaction (**VII**) is reacted with ethyl lithium to give (**VIII**), which is then isomerized to the *cis* form by ultraviolet irradiation in the presence of the photosensitizer anthracene [20]. Finally, the hydroxyl groups are deprotected with tetrabutylammonium fluoride in tetrahydrofuran to give (**IX**). A Wittig reaction with (**VI**) is also a step in the synthesis of the antipsoriatic drug MC 903 (calcipotriol, see Table III) [18].

Another route to new analogs involves $NaBH_4$ reduction of the aldehyde (**VI**), followed by tosylation of the resulting alcohol to give (**X**), which is subsequently reacted with a Grignard reagent to form (**XI**). Finally, (**XI**) is isomerized and deprotected to provide the analog CB 966 (**XII**) (see Table II) [21].

Analogs containing an oxygen atom in the 22 position (22-oxa analogs) can be synthesized as shown in Scheme 3, which depicts the synthesis of the C-20 epimeric compounds KH 1139 and KH 1060 (see Table IV) [22]. Oxidation of the aldehyde (**VI**) with air in the presence of a copper catalyst yields the methylketone (**XIII**), which on reduction with $NaBH_4$ forms a mixture of the two epimeric alcohols (**XIVa** and **XIVb**), where the 20-epi isomer (**XIVb**) dominates. Alkylation of the two alcohols, followed by isomerization
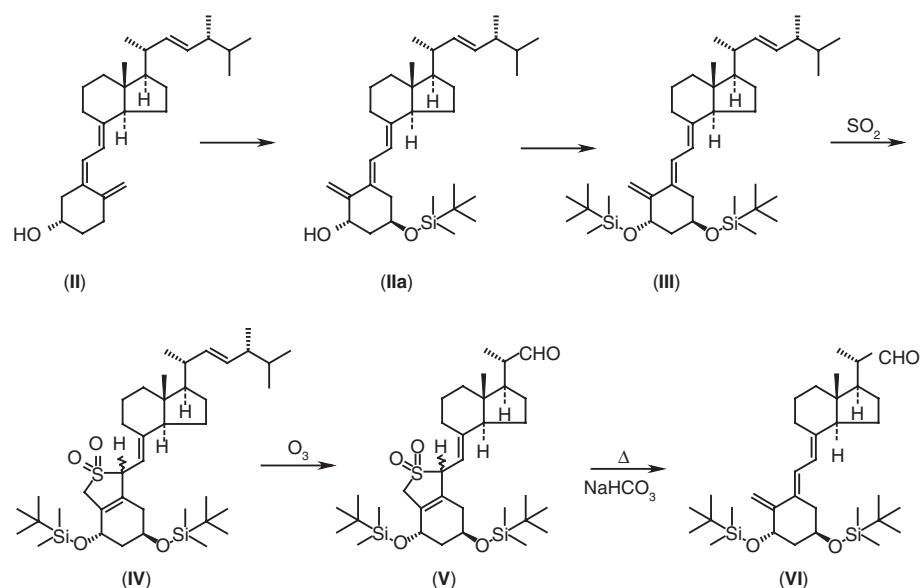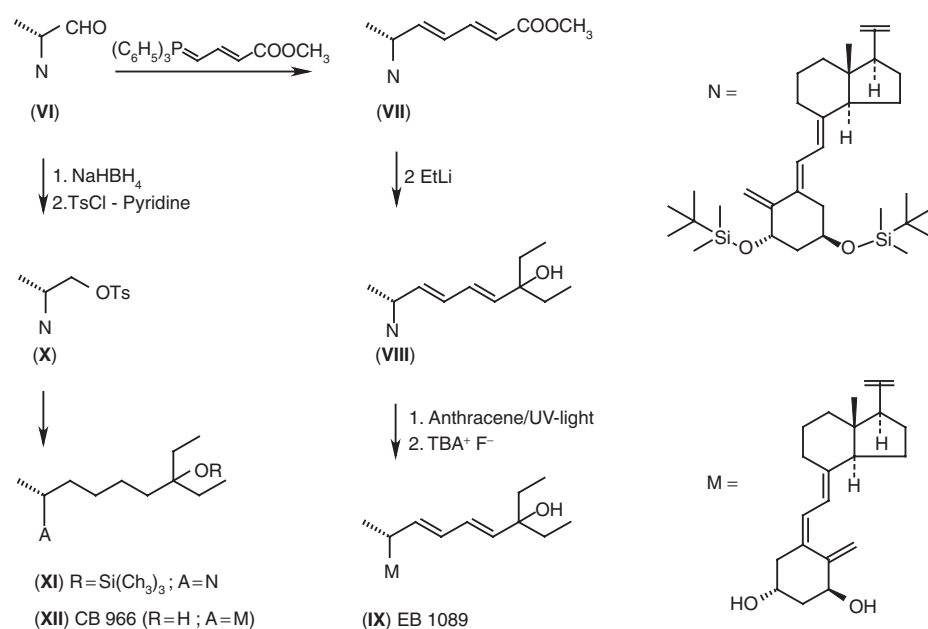
SCHEME 1

and deprotection as described above, yields the cis analogs (**XVa**) (KH 1139) and (**XVb**) (KH 1060), respectively. As described in Section III.D, epimerization at C-20 has a profound influence on the biological properties of the analogs.

Another route to 20-epi compounds (Scheme 3) starts with an epimerization of the aldehyde (**VI**) to the 20-epi aldehyde (**XVI**), which then, by the same sequence of reactions used in the 20-normal series (Scheme 2), can be converted to 20-epi analogs, such as 20-epi-1,25(OH)$_2$D$_3$ (**XVII**) (MC 1288) (see Table IV) [23]. The tosylate (**XVIII**), used in the synthesis of MC 1288, has also been used to synthesize the 20-epi-23-thia analog (**XIX**) (GS 1790) (see Table V) [24].

The reactions depicted in Schemes 1–3 are typical of the pathways used in the synthesis of a wide variety

SCHEME 2
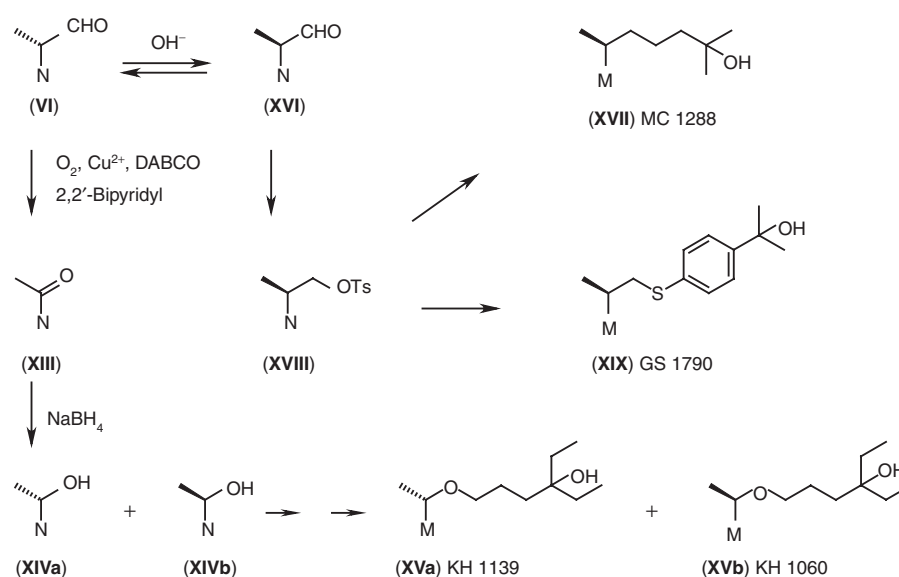
SCHEME 3   N and M have the same meaning as in Scheme 2.

of side chain analogs, but it is obvious that many variations have been necessary.

III. STRUCTURE-ACTIVITY RELATIONSHIPS

In this section, the effect of systematic chemical modifications of the $1,25(OH)_2D_3$ side chain on various biological parameters is presented. All the analogs discussed here have been tested for calcemic activity, antiproliferative activity, and ability to induce cell differentiation, as described in Section II.A. However, because the antiproliferative and differentiation-inducing properties run parallel, only the antiproliferative potencies are shown in the tables. All values are given in relation to $1,25(OH)_2D_3$.

A. Variation of Chain Length

In Table I the biological activities of a number of analogs which differ from $1,25(OH)_2D_3$ with respect to the length of the C-17 side chain are listed [21]. It is seen that if the chain length is increased with one methylene group as in MC 1127, the ability to inhibit proliferation is increased, whereas the calcemic activity is reduced to about one-third of that of $1,25(OH)_2D_3$. This compound has also been described by Ostrem *et al.* [25]. If the two terminal methyl groups in MC 1127 are replaced by ethyl groups as in CB 966, the antiproliferative potency is increased
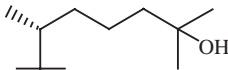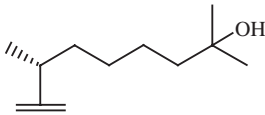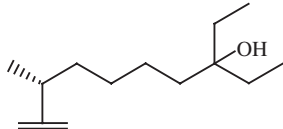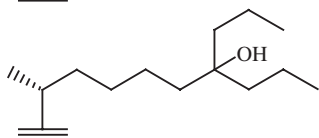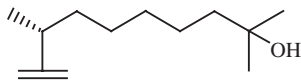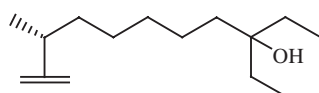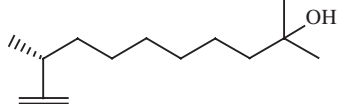
and the calcemic activity further reduced. On the other hand, if propyl groups are substituted for the methyl groups, the antiproliferative potency is reduced to about the same level as that of $1,25(OH)_2D_3$. An increase of the $1,25(OH)_2D_3$ side chain with two methylene groups (MC 1147), also described by Kutner *et al.* [26], causes a further reduction of the calcemic activity, whereas the antiproliferative potency remains the same as in MC 1127. However, with the introduction of one more methylene group (MC 1179), the antiproliferative activity is reduced. In other words, the optimal number of methylene groups between C-20 and the tertiary hydroxyl group seems to be four or five.

B. Introduction of Double and Triple Bonds

The effect of introducing one or two double bonds in the C-17 side chain is illustrated in Table II. Whereas the introduction of a Δ^{22} double or triple bond in CB 966 decreases the antiproliferative activity [23,27], the introduction of a further Δ^{24} double bond leads to EB 1089 [19], which, with respect to cell proliferation, is the most active in the series, being 100 times more potent than $1,25(OH)_2D_3$. Because the calcemic activity of EB 1089 is three times lower, a substantial separation of the effects has been achieved. EB 1089 has been chosen as candidate for clinical testing in cancer patients (see Section V.B.2).

Another analog, CB 1093, is characterized by several modifications in the side chain. In addition to the triple bond, CB 1093 has altered stereochemistry at

TABLE I   Variation of Chain Length

Compound	Side chain structure	Inhibition of U937 cell proliferation IC$_{50}$ (M)	Calcemic activity relative to 1,25(OH)$_2$D$_3$ (%)
1,25(OH)$_2$D$_3$		3×10^{-8} [1X][a]	100
MC 1127		5×10^{-9} [6X]	38
CB 966		1×10^{-9} [30X]	17
CB 973		5×10^{-8} [0.6X]	n.d.[b]
MC 1147		5×10^{-9} [6X]	4
CB 953		2×10^{-8} [1X]	2
MC 1179		4×10^{-8} [0.7X]	2

[a]Boldface figures indicate activity relative to 1,25(OH)$_2$D$_3$.
[b]Not determined.

C-20 and an ethoxy group at C-22. These modifications increase the antiproliferative activity of the compound to the same level as that of EB 1089. CB 1093 has recently been shown to be a potent inducer of apoptosis (see Section IV.C).

The last compound in Table II, HEP 187, is a 20-epi-vitamin D analog, in which the terminal side chain hydroxy group has been replaced by a fluorine atom. This compound has a relatively low antiproliferative effect, compared to the other 20-epi-analogs, but it has been shown to exert interesting effects on bone mineral metabolism (see Section IV.E.3).

C. Calcipotriol and Related Analogs

One of the first 1,25(OH)$_2$D$_3$ analogs synthesized at LEO for which a clear separation between the calcemic activity and the effects on cell regulation was achieved was MC 903 [10,18] (Table III), which later received the United States Adopted Name (USAN) calcipotriene and the International Nonproprietary Name (INN) calcipotriol. In this compound, a Δ^{22} double bond is introduced in the side chain, the 25-hydroxyl is moved to the 24 position (with the indicated stereochemistry), and a cyclopropane ring is substituted for the isopropyl group in 1,25(OH)$_2$D$_3$. Table III shows that calcipotriol has retained the cell-regulating potency of 1,25(OH)$_2$D$_3$, whereas its calcemic activity has been reduced by a factor of 200.

As described in Sections IV.E.2 and V.B.1 of this chapter and in Chapter 101, calcipotriol has become an important antipsoriatic drug. Its 24-epimer MC 900 [18] has a considerably lower antiproliferative potency, and the same holds true for MC 1046 and MC 1080, the two main metabolites of calcipotriol [28].

TABLE II Double and Triple Bonds

Compound	Side chain structure	Inhibition of U937 cell proliferation IC$_{50}$ (M)	Calcemic activity relative to 1,25(OH)$_2$D$_3$ (%)
1,25(OH)$_2$D$_3$		3×10^{-8} [1X][a]	100
CB 966		1×10^{-9} [30X]	17
MC 1473		2×10^{-8} [1.5X]	24
CB 1309		3×10^{-8} [1X]	n.d.[b]
EB 1089 (seocalcitol)		3×10^{-10} [100X]	31
CB 1093		3×10^{-10} [100X]	24
HEP 187		5×10^{-9} [6X]	34

[a]Boldface figures indicate activity relative to 1,25(OH)$_2$D$_3$.
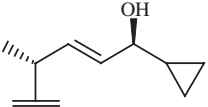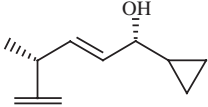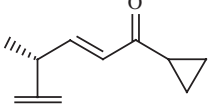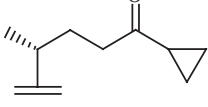[b]Not determined.
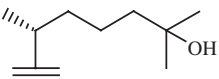
The effect of the size of the terminal ring was studied by Calverley [29], who synthesized three pairs of 24-epimeric analogs in which the cyclopropyl group in calcipotriol is replaced by cyclobutyl, cyclopentyl, and cyclohexyl groups, respectively. Although the stereochemistry of the 24-hydroxyl group in these compounds has not been rigorously established, their polarities suggest that in MC 1070, MC 1052, and MC 1048 the 24-hydroxyl group has the same stereochemistry as in calcipotriol, whereas in MC 1069, MC 1050, and MC 1033 the stereochemistry of the 24-hydroxyl group is as in MC 900. It was found that the antiproliferative potencies of the cyclobutyl and cyclopentyl analogs MC 1070 and MC 1050 were similar to that of calcipotriol, whereas the cyclohexyl analog MC 1048 was less potent. All these compounds were, however, significantly more calcemic than calcipotriol.

D. Epimerization at C-20

A seemingly minor modification of the side chain in 1,25(OH)$_2$D$_3$ that has a dramatic effect on its biological activities is epimerization at C-20. As Table IV shows, the 20-epimer of 1,25(OH)$_2$D$_3$ (MC 1288) [23] is about 100 times more potent than the natural hormone as an inhibitor of cell proliferation, whereas its calcemic activity has increased by a factor of only 2. Even more pronounced is the effect of 20-epimerization on immunosuppressive properties [30].

In view of these results, we found it mandatory to investigate the effect of 20-epimerization more broadly [22,23]. Table IV shows the activities of pairs of 20-epimers. Both the antiproliferative potency and the calcemic activity are generally higher in the 20-epi than in the 20-normal series. A particularly noteworthy compound is the 22-oxa analog KH 1060 (lexacalcitol),

TABLE III   Calcipotriol and Analogs

Compound	Side chain structure	Inhibition of U937 cell proliferation IC$_{50}$ (M)	Calcemic activity relative to 1,25(OH)$_2$D$_3$ (%)
1,25(OH)$_2$D$_3$		3×10^{-8} **[1X]**[a]	100
MC 903 (calcipotriol)		2×10^{-8} **[1.5X]**	0.5
MC 900		$>1 \times 10^{-7}$ **[<0.3X]**	<1
MC 1046		$>1 \times 10^{-7}$ **[<0.3X]**	<1
MC 1080		$>1 \times 10^{-7}$ **[<0.3X]**	<1

[a]Boldface figures indicate activity relative to 1,25(OH)$_2$D$_3$.

which is 3000 times more potent than 1,25(OH)$_2$D$_3$ as a proliferation inhibitor but only slightly more calcemic.

E. Introduction of an Aromatic Ring

The effect of introducing a benzene ring in the side chain is illustrated in Table V. The analogs are all 20-epi compounds, where the benzene ring is attached to an oxygen or a sulfur atom in the 23 position and further substituted in the ortho, meta, or para position with a 2-hydroxypropyl or a 3-hydroxypentyl group [24]. Whereas the ortho- and para-substituted analogs have antiproliferative potencies of the same order of magnitude as 1,25(OH)$_2$D$_3$, the meta-substituted analogs (EB 1213, EB 1219, GS 1500, and GS 1730) are considerably more potent. All the compounds tested are at least one order of magnitude less calcemic than 1,25(OH)$_2$D$_3$. From this series of analogs, GS 1790 has recently been selected as an interesting candidate for preclinical studies in experimental models of bone disease (see Section IV.E.3).

IV. BIOLOGICAL ACTIVITIES

During the last 10 years, research on the effects of 1,25(OH)$_2$D$_3$ at the cellular and molecular level has

intensified, partly due to the availability of new synthetic analogs. The new findings have led to an increased understanding of the therapeutic potential of the analogs, of which a substantial number has been investigated *in vivo*, both in experimental animal models and in clinical trials.

This section describes the main biological findings with a number of LEO analogs in the fields of receptor activated gene expression, cell growth and regulation, immune regulation, and bone mineral metabolism.

A. VDR Binding and Gene Expression

Most of the biological effects of 1,25(OH)$_2$D$_3$ *in vitro* and *in vivo* are believed to be mediated via the VDR, but the activity of many vitamin D analogs is not directly correlated with their binding affinity for the receptor. The prevailing theory has been that different analogs may confer different conformational changes to the VDR ligand complex. This may in turn lead to altered stability of the complex and/or an altered ability to interact with DNA-binding sites or protein coactivators and corepressors.

The use of vitamin D analogs with altered stereochemistry at C-20, the 20-epi-vitamin D analogs, has allowed the investigation of the relative importance of

TABLE IV   Effect of 20-Epimerization

Compound	Side chain structure	Inhibition of U937 cell proliferation IC$_{50}$ (M)	Calcemic activity relative to 1,25(OH)$_2$D$_3$ (%)
1,25(OH)$_2$D$_3$		3×10^{-8} **[1X]**[a]	100
MC 1288		3×10^{-10} **[100X]**	200
MC 1127		5×10^{-9} **[6X]**	38
EB 1231		6×10^{-11} **[500X]**	500
CB 966		1×10^{-9} **[30X]**	17
MC 1301		3×10^{-10} **[100X]**	200
KH 1139		2×10^{-10} **[150X]**	30
KH 1060 (lexacalcitol)		1×10^{-11} **[3000X]**	130

[a]Boldface figures indicate activity relative to 1,25(OH)$_2$D$_3$.

the various factors that may increase the transcriptional activity of the VDR. Studies on the direct interaction between the VDR and a tritium-labeled 20-epi-analog ([1-^{3}H]GS 1500) showed that GS 1500 (see Table V) bound to the same binding site as 1,25(OH)$_2$D$_3$, but was able to induce the VDR to form a DNA-binding complex at a concentration 100 times lower than that of 1,25(OH)$_2$D$_3$ [31].

Further studies with the biologically very potent 20-epi-analogs MC 1288 and KH 1060 have suggested that these compounds increase the stability of the analog-VDR complex [32–34] by making it more resistant to protease degradation than that of the natural ligand 1,25(OH)$_2$D$_3$ [35,36]. This increased stability has been linked to the induction of conformational changes in the VDR that prevent binding of the proteins involved in the ubiquitin/proteasome mediated degradation of the receptors [37,38].

The recent availability of the crystal structures of the ligand-binding domain of the VDR, complexed to 1,25(OH)$_2$D$_3$ or to the 20-epi-analogs MC 1288 and KH 1060, has permitted a more detailed investigation of the way in which the VDR interacts with agonistic compounds. The results indicate that both 1,25(OH)$_2$D$_3$ and the analogs adapt to the binding pocket, with new points of contact to the protein, but without inducing major conformational changes [39]. Conformational changes may thus be of less importance than previously

TABLE V   Analogs with an Aromatic Ring

Compound	Side chain structure	Inhibition of U937 cell proliferation IC$_{50}$ (M)	Calcemic activity relative to 1,25(OH)$_2$D$_3$ (%)
1,25(OH)$_2$D$_3$		3×10^{-8} [1X][a]	100
EB 1224		2×10^{-8} [1.5X]	3
EB 1213		1×10^{-10} [300X]	7
EB 1220		8×10^{-8} [0.3X]	n.d.[b]
EB 1219		8×10^{-11} [375X]	6
GS 1780		6×10^{-8} [0.5X]	<1
GS 1500		1×10^{-10} [300X]	<1
GS 1790		5×10^{-8} [0.6X]	<1
GS 1730		1×10^{-9} [30X]	<1

[a]Boldface figures indicate activity relative to 1,25(OH)$_2$D$_3$.
[b]Not determined.

thought, and the increased transcriptional activity of the analogs may be primarily related to the increased stability and half-life of the ligand-receptor complex [39,40].

Another consequence of the increased stabilization induced by vitamin D analogs may be enhanced dimerization with RXR or increased recruitment of coactivators that facilitate interaction between nuclear receptors and the basal transcription machinery. In this respect, a close correlation between the ability of MC 1288 to induce cell differentiation, p21 transactivation, and recruitment of the coactivator protein complex DRIP to the VDR has been observed [41]. Very recent studies have shown that the strength of interaction of coactivators such as GRIP1 and RAC3 with the VDR is enhanced by the 20-epi-analogs MC 1288, KH 1060, and MC 1301, but not by analogs with 20-normal configuration [42]. The interaction of VDR with coactivators may lead to opening of the chromatin by activation of histone acetylases

and permit transcription of vitamin D responsive genes [43].

The most complete analysis of vitamin D–induced gene expression in a single tissue has recently been performed with the 20-normal analog EB 1089. Human head and neck squamous carcinoma cells were incubated with or without the analog for up to 48 hrs and gene expression was studied using DNA microarray screening. More than 150 genes were regulated, including genes involved in cell cycle progression, cell differentiation, cell adhesion, extracellular matrix composition, and cellular functions of the immune system [44].

B. Cell Cycle Regulation

The ability of vitamin D analogs to inhibit proliferation and induce differentiation in a large number of different cell types has led to the development of several of these analogs for the treatment of hyperproliferative diseases such as psoriasis and cancer. One mechanism by which vitamin D compounds may exert their effects on cellular growth is by regulating cell cycle progression. Most cells respond to $1,25(OH)_2D_3$ or its analogs by growth arrest, associated with block in the G_0/G_1 phase [45]. Early studies indicated that growth inhibition of human keratinocytes was linked to dephosphorylation of pRb [46]. Since then, the study of the molecular basis for induction of cell cycle blockade by vitamin D compounds has been intensified. Up-regulation of the two cyclin-dependent kinase inhibitors p21[WAF1/CIP1] and p27[KIP1] appears to be one of the main mechanisms of the G_1-block.

The vitamin D analog EB 1089, which is 50–100 times more potent than $1,25(OH)_2D_3$ in inhibiting proliferation of a large number of cancer cell lines, has been used extensively in the study of cell cycle regulation, showing differential regulation of cyclin/cdk activity in various cancer cells. In HL-60 promyelocytic leukemia cells, EB 1089 induced p27[WAF1/CIP1] and decreased cdk2 and cdk6 activities [47], whereas in NCI-H929 myeloma cells, EB 1089 induced p27[KIP1] and reduced the cdk2, but not the cdk6 activity [48]. These findings were further refined by a study in thyroid carcinoma cells, where EB 1089 similarly induced expression of p27[KIP1] and also protected the cdk inhibitor from degradation, possibly by activation of phosphatases like PTEN [49].

A recent study has linked the effects of EB 1089 on cell proliferation directly to those on cell differentiation. Using SW480-ADH colon cancer cells, EB 1089 was shown to induce expression of E-cadherin and sequestration of β-catenin in the plasma membrane, leading to blockade of the β-catenin signaling pathway, down-regulation of genes involved in cell cycle regulation (such as cyclin D1 and c-myc), and induction of differentiation [50].

C. Apoptosis

Induction of growth arrest by vitamin D compounds is also linked to induction of apoptosis, especially in cancer cells. Apoptotic features have been observed in many types of cancer cells including breast, colon, and prostate cancer cells, after treatment *in vitro* with $1,25(OH)_2D_3$ or analogs. EB 1089 has been shown to be a more potent inducer of apoptosis than $1,25(OH)_2D_3$ in a study with 5 different colon cancer cell lines [51]. The apoptotic effects of EB 1089 could be increased by combination with TGFβ [52] or antiestrogens [53].

The mechanism(s) by which $1,25(OH)_2D_3$ and its analogs induce apoptosis may vary from one cell type to another, but in most studies induction of apoptosis has been found to be independent of the p53 tumor suppressor status and did not involve caspase activation [54]. Several studies have shown that both $1,25(OH)_2D_3$ and EB 1089 increase intracellular calcium levels, disrupt mitochondrial functions, and induce cytochrome c release, thus activating caspase-independent cell death [55,56]. This pathway may also include activation of the calcium-dependent cysteine protease, calpain [56]. In addition, EB 1089 has also been shown to down-regulate the antiapoptotic protein bcl-2 [57]. Recently, CB 1093, a vitamin D analog with 20-epi configuration and a triple bond in the side chain (see Table II), has also shown potent apoptosis-inducing effects [58,59].

A number of cell lines from tissues such as skin and bone are resistant to induction of apoptosis by vitamin D compounds. In particular, vitamin D analogs such as MC 903 and CB 1093 have been shown to display antiapoptotic effects in osteoblast-like cells [60]. These findings suggest that the ability of vitamin D compounds to stimulate bone formation may, to some extent, be linked to their ability to protect the osteoblasts from undergoing apoptosis.

D. Growth Factors

$1,25(OH)_2D_3$ and its analogs interfere with the mitogenic activity of growth factors, such as IGF-I, IGF-II, and EGF [61,62]. These effects are mainly seen in cancer cells with an increased growth factor expression or activity, which contribute to their survival. The analog EB 1089 was able to inhibit growth of IGF-I stimulated human breast cancer cells [63], and both

EB 1089 and CB 1093 were shown to inhibit the secretion of IGF-II in human colon cancer cells [64]. Blocking of the growth-stimulating activity of IGF-I has been associated with suppression of IGF-receptor 1 expression [61] and with up-regulation of IGF-binding proteins [65,66]. The IGFBPs control the availability of the growth factors, and both EB 1089 and CB 1093 up-regulate IGFBP-6 in colon cancer cells [64] and IGFBP-3 in breast cancer cells [67]. Another study has shown up-regulation of IGFBP-5 in breast cancer cells by EB 1089 and KH 1060 [68].

Vitamin D compounds also inhibit cell proliferation by interfering with signaling through the EGF receptor. A recent study shows that MC 903 is able to inhibit the autocrine phosphorylation of the EGF-receptor in squamous carcinoma cells, resulting in a potent inhibition of cell growth and induction of differentiation [69].

In contrast, treatment of hyperproliferative epithelial cells with $1,25(OH)_2D_3$ often results in an increased secretion of TGFβ or an enhanced expression of TGFβ receptors. In these cells, TGFβ acts as a negative growth regulator and up-regulation of TGFβ activity results in growth inhibition. The analogs EB 1089 and MC 903 enhanced TGFβ_1 expression and protein secretion in breast cancer cells [70], whereas other studies showed that EB 1089 was able to up-regulate TGFβ receptor II in breast cancer cells [71] and TGFβ receptors I and II in HL-60 myeloid leukemia cells [72].

The role of vitamin D analogs in regulating growth factor expression and proliferation of epithelial cells is an important new area of research, with implications for the treatment of cancer and other hyperproliferative diseases.

E. Preclinical Experience with Vitamin D Analogs

1. CANCER MODELS

The rationale for the use of vitamin D analogs in cancer is based on the findings described previously in this chapter and more fully discussed in Chapters 89–97 of this book. The search for new analogs with potential clinical usefulness has been directed at finding a candidate with a good systemic bioavailability, potent effects on cell proliferation, an ability to activate apoptotic pathways, and, very importantly, a reduced calcemic activity compared to $1,25(OH)_2D_3$. From our screening program, we selected the analog EB 1089 (seocalcitol), which fulfilled these criteria. EB 1089 is characterized by having two double bonds in the side chain, and methyl groups at C26 and C27, which makes it less susceptible to metabolic degradation (see Table II).

The anticancer effects of EB 1089 have been studied in numerous experimental animal studies. Colston *et al.* were the first to show that EB 1089 inhibited tumor growth *in vivo* [73]. Rats with nitrosomethylurea (NMU)-induced mammary tumors were treated with EB 1089 at 0.5 µg/kg/day p.o. for 4 weeks. The treatment induced a significant inhibition of tumor growth, without changes in the serum calcium levels. In contrast, treatment with the same dose of $1,25(OH)_2D_3$ did not affect tumor growth but caused hypercalcemia. Interestingly, histological analysis of NMU-induced tumors from rats treated with EB 1089 has shown evidence of apoptotic cell death, with large areas exhibiting loss of cellularity, a low mitotic index, and nuclear DNA fragmentation [74]. Similar results have been obtained in nude mice with breast cancer xenografts treated with EB 1089 [75]. Further support for the potential role of EB 1089 in the treatment of breast cancer came from a study showing that EB 1089 was able to inhibit the development of osteolytic bone metastases from mammary tumors in nude mice [76]. Other studies have shown that EB 1089 can be effectively combined with taxol, tamoxifen, or anti-estrogens [77–79]. In addition, recent findings suggest that EB 1089 may also be active against estrogen-resistant tumors [53].

Another area of interest has been the potential usefulness of EB 1089 in the treatment of prostate cancer. $1,25(OH)_2D_3$ is able to inhibit the growth of human prostate cancer cell lines *in vitro*, and pilot studies in patients undergoing surgery for prostate cancer have shown that $1,25(OH)_2D_3$ may decrease the rate of rise of the prostate specific antigen, indicating an effect of the treatment on the rate of recurrence of the disease [80]. The analog EB 1089 is a potent inhibitor of prostate cancer cell proliferation *in vitro* and induces differentiation or apoptosis in several of these cell lines [81–83]. *In vivo*, EB 1089 inhibited the growth of androgen-resistant metastatic prostate cancer cells in rats and reduced the number of lung metastases with significantly less calcemic toxicity than $1,25(OH)_2D_3$ [84]. Another study has shown that EB 1089 also inhibits prostate cancer xenografts in nude mice, resulting in tumors significantly less vascularized than in control animals [85]. However, no effects were seen with EB 1089 in a study designed to prevent prostate cancer in a model of transgenic mice which develop androgen resistant prostate cancer [86].

Despite the encouraging preclinical evidence obtained with EB 1089 in the animal models described above, broad clinical testing with this analog has not been initiated in patients with breast and prostate cancers. As will be described in the next section, studies on the pharmacokinetic profile and tissue distribution of EB 1089 have indicated that administration of this analog leads

to very high concentrations of active compound in specific tissues, especially in the liver. The recent preclinical and clinical development of EB 1089 has therefore been targeted toward indications such as hepatocellular carcinomas and colon cancer with risk of liver metastasis. In this regard, it is relevant to note that EB 1089 has been shown to inhibit human colon cancer cell xenografts in nude mice [87] and to potently reduce the incidence of spontaneous hepatocellular carcinomas in mice [88].

2. IMMUNOLOGICAL DISEASES

Besides its classic actions on calcium metabolism and its effects on cell proliferation and differentiation, $1,25(OH)_2D_3$ also modulates a number of immunological functions (see Chapters 98 and 99). The VDR is constitutively expressed in most cell types of the immune system, especially in antigen presenting cells. In T-lymphocytes, up-regulation of the VDR occurs in response to $1,25(OH)_2D_3$ [89]. $1,25(OH)_2D_3$ inhibits antigen-induced T-lymphocyte proliferation and cytokine production, mainly by targeting the subset of T-helper 1 (TH1) cells that preferentially produce IL-2 and interferon-γ (IFN-γ) [90–92].

The rationale for development of vitamin D analogs for psoriasis has been based on the ability of analogs such as calcipotriol to inhibit hyperproliferative skin cells and normalize their aberrant pattern of differentiation, and also on their immune regulatory effects on TH1-type lymphocytes that are implicated in the pathogenesis of this autoimmune disease [93,94].

The beneficial effect of vitamin D analogs on the regulation of the immune system may be enhanced by combination with immunosuppressive agents like cyclosporine A or steroids. A recent study has shown additive effects of combinations of $1,25(OH)_2D_3$ and steroids on the suppression of T-lymphocyte proliferation and INF-γ production, whereas TH2-type responses were not inhibited [95]. Very recently, another study has provided evidence for a synergistic effect of combined steroid and vitamin D treatment. Using $1,25(OH)_2D_3$ and dexamethasone, Barrat *et al.* [96] have shown that combined treatment induced the appearance of regulatory T-cells producing IL-10, but not TH1-type cytokines. These regulatory T-cells were able to suppress inflammation in an animal model of autoimmunity.

Such findings provide the basis for the development of a new type of topical therapy for psoriasis. In this regard, calcipotriol, in combination with betamethasone dipropionate, has recently been shown to have a more effective antipsoriatic activity than either of the agents alone (see Section V.B.1).

Besides the use of topical vitamin D analogs in skin diseases, the discovery of the very potent analogs with

20-epi side chain configuration has led to a marked interest in their potential use for systemic treatment of graft rejection and autoimmune diseases [30]. Extensive studies have been performed in animal models, in particular with the two 20-epi analogs KH 1060 and MC 1288. MC 1288, 20-epi-$1,25(OH)_2D_3$, was selected as our candidate for studying the potential of this class of agents in models of graft rejection. MC 1288, at 0.1 μg/kg/day i.p., was shown to prolong the survival of cardiac allografts in rats, with effects comparable to those obtained with cyclosporine A [97,98]. In the same model, combined treatment with MC 1288 and cyclosporine A produced a significantly prolonged survival time of the cardiac grafts, compared with therapy with either agent alone [99]. In contrast, treatment with MC 1288 alone was not able to prevent rejection of mouse-to-rat cardiac xenotransplants, unless combined with low-dose immunosuppressive therapy [100].

MC 1288 has also been studied for its effects on chronic rejection (allograft arteriosclerosis) using aortic allografting in rats [101]. Chronic rejection is the single most important reason for late graft loss. Changes include adventitial inflammation and intimal thickening. Both parameters were suppressed by long-term treatment with MC 1288, in combination with low-dose cyclosporine A.

In order to evaluate the potential of MC 1288 as treatment for human transplantation patients, a preclinical study of kidney transplantation in rhesus monkeys was undertaken by LEO (LEO internal report). MC 1288 was administered at the highest possible dose that did not affect serum calcium levels. No significant changes in graft survival time were observed in monkeys treated with MC 1288 as compared to untreated animals. No differences in the pattern of T-lymphocyte subsets or IL-2 serum levels were observed. It was concluded, from the negative results of this study, that monotherapy of graft rejection with vitamin D analogs was not indicated, and that further efforts should be directed toward combination therapy with classic immunosuppressive agents.

The 20-epi analogs have also been investigated for immune intervention in other disease areas. KH 1060 was shown to prevent autoimmune type I diabetes in NOD mice [102] and to delay recurrence of diabetes in NOD mice with syngeneic islet grafts [103]. Subsequent use of the analog MC 1288 in the more advanced stage of disease in NOD mice showed that this analog was not able to reduce the incidence of overt diabetes when treatment was delayed until after the onset of insulitis [104]. When treatment with MC 1288 was combined with a short induction course of cyclosporine A, more than 50% reduction in diabetes incidence was seen [104].

Other studies with the 20-epi-analogs have shown positive effects in experimental autoimmune encephalitis

in mice [105], suppression of arthritis in rats with collagen-induced arthritis [106], and inhibitory effects on T-lymphocyte proliferation in cells obtained from patients with ulcerative colitis [107].

Taken together, these studies show that vitamin D analogs regulate autoimmune responses of the TH1-type in different disease settings, and also that systemic administration of clinically effective dosages is limited, due to increases in calcium levels. Combination with classic immunosuppressive agents may enhance efficacy and reduce the risk of side effects of both types of administered drugs.

3. BONE DISEASES

The classic physiological role of $1,25(OH)_2D_3$ is to regulate calcium homeostasis and promote bone mineralization. The role of $1,25(OH)_2D_3$ in the treatment of bone diseases such as rickets and renal osteodystrophy is well established, and several reports indicate that $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$ may also be beneficial for patients with osteoporosis [108,109].

Surprisingly, efforts to develop new synthetic vitamin D analogs for the treatment of bone diseases have been modest over the past years, but with the event of new analogs (maxacalcitol, paricalcitol) for the treatment of hyperparathyroidism and renal osteodystrophy, interest in the treatment of metabolic bone diseases with synthetic analogs seems to have returned. This interest has mainly been focused on two pathways: the design of analogs with modified pharmacokinetic and metabolic profiles and the design of tissue-specific analogs, along the lines that are currently used in the development of estrogen receptor agonists and antagonists.

The bone forming cells, the osteoblasts, are the primary target for vitamin D analog therapy. Osteoblastic cell lines express the VDR, whereas the bone resorbing cells, the osteoclasts, have long been considered not to possess VDR. A recent study using human bone biopsy samples has, however, detected expression of VDR in a percentage of osteoclasts and stromal cells, suggesting that these cells may also be direct targets for VDR agonists [110].

Previous studies have shown that a number of the 20-epi-analogs (MC 1288, KH 1060, and CB 1093) potently stimulate the production of type I procollagen and osteocalcin from cultured osteoblastic cell lines [33,111,112]. In addition, a number of these vitamin D analogs have been found to increase the sensitivity of the osteoblasts to treatment with estrogens, as measured by stimulation of the creatine kinase B activity in ROS 17/2.8 cells [113,114]. These findings support a role for the combination of vitamin D compounds and estrogens in the treatment of postmenopausal osteoporosis.

Further understanding of the effects of vitamin D analogs on osteoblast functions comes from a recent study, in which the ability of several vitamin D analogs (MC 1288, EB 1089, CB 1093, and KH 1060) to induce apoptosis in human osteoblast-like MG-63 cells was investigated [60]. No induction of apoptosis was seen, but some of the analogs exerted antiapoptotic effects in MG-63 cells treated with known apoptosis-inducing agents. These findings suggest that the ability of vitamin D compounds to stimulate bone formation *in vivo* may, to some extent, be linked to their ability to protect the osteoblasts from undergoing apoptosis.

Animal models provide evidence of the ability of $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$ to stimulate bone formation and to improve bone mass in animals with experimental osteoporosis. In normal rats, short-term treatment with $1,25(OH)_2D_3$ depressed osteoclast numbers, augmented osteoblast recruitment, and increased bone mass [115]. In ovariectomized rats, a model of estrogen-depletion induced bone loss, $1,25(OH)_2D_3$ dose-dependently increased bone mass, but also increased serum calcium levels [116].

Two new vitamin D analogs have recently been investigated for their ability to prevent bone loss and to increase bone strength in ovariectomized rats. The first of these analogs, HEP 187, is a 20-epi vitamin D analog, in which the side chain hydroxy group that interacts with the VDR, has been replaced by a fluorine atom (Section III, Table II). The introduction of the fluorine atom is intended to increase the metabolic stability of the 20-epi side chain. *In vitro*, HEP 187 dose-dependently increased secretion of osteocalcin and type I procollagen from MG-63 osteoblast-like cells. HEP 187 was tested *in vivo* in ovariectomized rats, using oral administration of 0.1 and 1.0 μg/kg/day. Treatment increased bone mineral content and bone strength to the same extent as $1,25(OH)_2D_3$, but with lower calcemic effects [117]. Further screening has led to the identification of our present lead compound, GS 1790 (Section III, Table V). GS 1790 is a 20-epi-analog with an aromatic ring in the side chain and a sulfur atom at the 23 position. *In vitro*, GS 1790 potently increased the production of osteocalcin and type I procollagen. In the ovariectomy model, GS 1790 was administered orally at doses from 10 to 50 μg/kg/day for 7 weeks to aging rats. pQTC scanning showed a significantly increased total and trabecular bone mineral density and biomechanical tests showed an increased bone strength (Table VI). In contrast to the rats treated with $1,25(OH)_2D_3$, which showed a reduced local bone mineralization, GS 1790 promoted bone formation and bone strength with normal mineralization patterns and lower calcemic effects [118].

Recently, some fascinating studies have been performed with the vitamin D analog EB 1089 on the effect

TABLE VI   Effects of GS 1790 in Ovariectomized Rats

Test compound	Dosage (p.o)	Serum calcium mmol/l	Trabecular density mg/cm^3	Breaking strength N
Vehicle (non-OVX)	—	2.55	324.8[b]	290.8
		(0.10)	(93.6)	(90.7)
Vehicle (OVX control)	—	2.57	157.6	187.7
		(0.09)	(33.5)	(52.6)
1,25(OH)$_2$D$_3$ (OVX)	0.05 µg/kg	2.91[b]	290.8[a]	318.9[a]
		(0.10)	(65.4)	(60.7)
GS 1790 (OVX)	10 µg/kg	2.62	242.2	323.6[a]
		(0.17)	(49.7)	(70.8)
GS 1790 (OVX)	25 µg/kg	2.69	297.7[a]	327.3[a]
		(0.06)	(82.9)	(56.9)

Female Sprague Dawley rats were treated once daily for 28 days.

Trabecular density was measured in the proximal tibia and bone breaking strength in the L3 vertebral body.

[a]P < 0.05 vs. OVX control. [b]P < 0.01 vs. OVX control (Kruskall-Wallis followed by Dunn's test).

of microgravity on bone cells. Long-term exposure to microgravity, as experienced by astronauts, leads to a substantial loss of bone mass, and a NASA supported program is evaluating potential candidates for bone protection during space flights. In one study, EB 1089 has been shown to selectively stimulate osteoblastogenesis, but not osteoclastogenesis, in bone marrow cell cultures from hind limb suspended rats [119]. Another study with osteoblast-type cells grown in a simulated microgravity environment has shown that treatment with EB 1089 is able to reverse the defects in cell differentiation and VDR expression induced by microgravity conditions [120].

The use of new vitamin D analogs in the treatment of a variety of bone disorders thus seems to attract new attention. The discovery of selective effects of the analogs on bone cell growth, differentiation, and apoptosis, together with new information on signal transduction pathways and genes involved in the regulation of calcium metabolism makes this an attractive area for further investigation.

V. CLINICAL DEVELOPMENT OF LEO ANALOGS

A. Pharmacokinetics and Metabolism

The research and development program for vitamin D analogs at LEO has yielded a number of candidates for the treatment of skin diseases, cancer, immunological disorders, and bone diseases. Such candidates must meet specific pharmacological requirements, and they must also have a specific pharmacokinetic/metabolic profile, suitable to the intended route of administration. To minimize the risk of discontinuation due to a poor pharmacokinetic profile [121], investigations of the pharmacokinetic/metabolic properties of drug candidates are performed early in the development program, so that the profile of the compound may be optimized both with regard to efficiency and to safety. The major challenge is related to the bioanalytical capabilities, as vitamin D analogs are active at very low doses, and the concentrations in biological matrices are correspondingly very low. However, with the introduction of new generations of triple quadropole mass spectrometers, it is now possible to quantify vitamin D analogs in the lower pg/mL serum range [122].

In the following, the importance of the pharmacokinetic profile of vitamin D compounds is illustrated, using calcipotriol (MC 903) and seocalcitol (EB 1089) as examples.

1. CALCIPOTRIOL

Calcipotriol has now been used for more than 10 years as an efficacious and safe topical treatment of psoriasis. Investigations of the systemic pharmacokinetics of calcipotriol have shown that calcipotriol is very rapidly eliminated. After intravenous administration to rats, calcipotriol had a serum half-life of less than 10 min., whereas 1,25(OH)$_2$D$_3$ had a half-life of 2.3 hours [123]. Thus, the area under the serum level/time curve (AUC) was more than 100 times lower for calcipotriol than for 1,25(OH)$_2$D$_3$. Using the postmitochondrial fraction from livers from rats, minipigs, and humans, the two major metabolites of calcipotriol,

MC 1046 and MC 1080 (see Table III), were identified. The metabolic pathway involved oxidation at carbon 24 in the side chain and reduction of the Δ^{22} double bond. Formation of the 24-oxidized metabolites was found to constitute a deactivation pathway for calcipotriol, as the biological effects of MC 1046 and MC 1080 on cell proliferation and differentiation were much weaker than those exerted by calcipotriol (see Table III). The rapid systemic elimination due to the formation of metabolites with a low biological activity explains, at least partly, the very low calcemic activity of calcipotriol. In contrast to the rapid systemic metabolism, the metabolism of calcipotriol in different human keratinocytes (HPKA1 and HaCaT cells lines) and *in vivo* in rat skin was found to be very slow [124,125]. Therefore, calcipotriol has the ideal pharmacokinetic/metabolic profile for a topical drug, being metabolically stable at the target site to exert the maximal therapeutic effect, and thereafter being metabolized into inactive metabolites once it enters the systemic circulation, thus minimizing the risk of side effects.

2. SEOCALCITOL

Important parameters in the selection of the vitamin D analog seocalcitol as a candidate for the treatment of cancer have been its bioavailability and metabolic stability.

In vivo pharmacokinetic studies in rats and *in vitro* studies in postmitochondrial liver fraction from rats, minipigs, and humans have shown that seocalcitol has a half-life in serum comparable to that of $1,25(OH)_2D_3$ and that the analog is metabolically very stable [126]. Although the serum half-lives of seocalcitol and $1,25(OH)_2D_3$ are comparable, their apparent volumes of distribution are very different, as demonstrated by large differences in their serum concentrations after intravenous administration of the same dose [14]. This is explained by the difference in their binding affinity for the serum vitamin D–binding protein (DBP), for which $1,25(OH)_2D_3$ has a 30-fold higher binding affinity. Thus, the tissue distribution of the two compounds is different. From the autoradiogram in Fig. 1 it can be seen that seocalcitol is accumulated in the

liver after a single oral dose to a rat, with concentrations in the liver about 10-fold higher than in serum. Similar results were found in minipigs after oral administration of seocalcitol [127].

Due to the lack of a sensitive bioanalytical assay, very few pharmacokinetic studies with seocalcitol have been performed in humans. However, a pharmacokinetic study was performed in 84 healthy subjects, who were given a single oral dose of 15 µg seocalcitol as enterocoated capsules. The serum concentration versus time profile was characterized by a steady absorption following the lag phase (2 hours). The maximal serum concentration (C_{max}) was 57 pg/mL, and it was achieved at a median of 5 hours. The serum half-life was 14 hours (LEO internal report).

Assuming that the ratio of the liver/serum concentration of seocalcitol in humans is similar to that in rats and minipigs, the maximal concentration of seocalcitol in the human liver is above 450 pg/g ($\sim 10^{-9}$M). As the IC_{50} for the antiproliferative effect of seocalcitol in many cancer cells is 10^{-10}–10^{-9}M (Table VII), the concentration of seocalcitol in the liver is expected to be sufficiently high to be clinically effective.

The metabolic stability and the high affinity of seocalcitol for the liver makes it suitable for systemic treatment, with the liver as an attractive target organ.
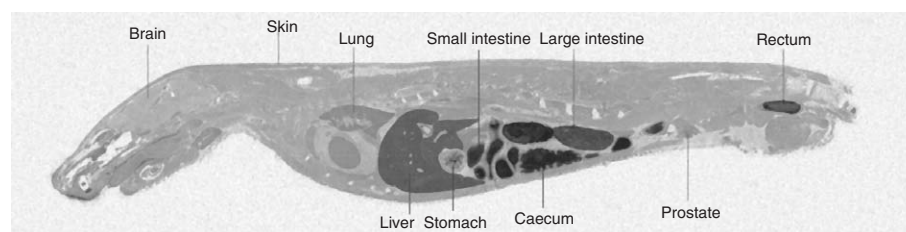
TABLE VII   Effects of EB 1089 (seocalcitol) on Tumor Cell Proliferation *In Vitro*

Cell line	Test compound	Inhibition of proliferation IC_{50} (M)
MCF-7 (human breast cancer)	EB 1089	2×10^{-10}
	$1,25(OH)_2D_3$	1×10^{-8}
	Daunomycin	2×10^{-8}
HT-29 (human colon cancer)	EB 1089	8×10^{-10}
	$1,25(OH)_2D_3$	4×10^{-8}
	Daunomycin	8×10^{-9}
B16 (mouse melanoma)	EB 1089	6×10^{-11}
	$1,25(OH)_2D_3$	6×10^{-9}
	Daunomycin	5×10^{-8}

FIGURE 1   Tissue distribution of seocalcitol in a rat 8 hours after oral administration of ^{3}H-seocalcitol (200 µg/kg).

B. EFFICACY AND SAFETY

1. PSORIASIS

Calcipotriol The rationale for the use of vitamin D analogs in the treatment of psoriasis is described in Section IV.E.2 and in Chapter 101. The efficacy and safety of topically applied calcipotriol to patients with mild to moderate psoriasis was established in double-blind, placebo-controlled studies initiated in 1987 [128,129]. Calcipotriol was launched in 1991, and numerous studies have since established the usefulness of this treatment of chronic plaque psoriasis (Table VIII). Calcipotriol is at least as effective as potent topical steroids, and is more effective than tazarotene (retinoid), short contact dithranol, and tar in the treatment of mild to moderate plaque psoriasis.

Combination Therapy Since the introduction of calcipotriol, the treatment of psoriasis has been further improved by combination therapy. A review of representative studies is included in Table VIII. Light therapy (PUVA and UVB) combined with calcipotriol seems to have a beneficial effect even at reduced UV doses. In moderate to severe psoriasis, calcipotriol has been used in combination with systemic treatments such as cyclosporine A, etretinate, methotrexate, or acitretin. In these studies, it was possible to reduce the dose of the systemic drug, thus reducing the risk of side effects. However, more long-term combination studies are required to evaluate the long-term risk-benefit ratio.

Calcipotriol has also been combined with various topical steroids, resulting in an improved efficacy compared to calcipotriol used as monotherapy.

Daivobet The beneficial effects of combining calcipotriol with steroids encouraged the development of the combination product Daivobet® (Table VIII). The rationale for this combination therapy is discussed in Section IV.E.2.

Four phase III studies including more than 4,500 psoriasis patients were performed with Daivobet ointment, containing 50 µg/g calcipotriol and 0.5 mg/g betamethasone diproprionate (Table VIII). Once daily application was well tolerated and was more effective than twice daily use of calcipotriol or steroid alone, with a more rapid onset of response.

2. CANCER

Seocalcitol The rationale for the use of vitamin D analogs in cancer is briefly described in Section IV.E.1, and more fully discussed in Chapters 89–97. A clinical program for the study of seocalcitol in the treatment of cancer was initiated in 1995. Dosing experience from both phase I and II studies indicated that 15 µg seocalcitol per day was a safe starting dose. The primary toxicity of the drug was associated with its calcemic properties, i.e. hypercalcemia and related clinical symptoms. Phase II studies in leukemia, breast cancer, and pancreatic cancer have been completed without observation of any complete or partial response.

TABLE VIII   Calcipotriol: Monotherapy and Combination Therapy of Psoriasis

Calcipotriol Monotherapy Comparative Studies	References
Comparative studies of calcipotriol and corticosteroids	Crosti *et al.* [138]; Molin *et al.* [139]; Bruce *et al.* [140]; Cunliffe *et al.* [141]; Kragballe *et al.* [142]
Comparative study evaluating efficacy and tolerability of calcipotriol and tazarotene in psoriatic lesions treatment	Kokelj *et al.* [143]
Comparative studies of calcipotriol and short contact dithranol in psoriasis vulgaris	Wall *et al.* [144]; Berth-Jones *et al.* [145]
A comparative study of calcipotriol ointment and tar in chronic plaque psoriasis	Tham *et al.* [146]
Calcipotriol in Combination Therapy Studies	
Comparative studies of calcipotriol in combination with PUVA	Youn *et al.* [147]; Frappaz *et al.* [148]
Comparative studies of calcipotriol in combination with UVB (broad and narrow band)	Ramsay *et al.* [149]; Molin [150]; Picot *et al.* [151]; Brands *et al.* [152]; Kerscher *et al.* [153]
Calcipotriol combined with systemic treatment (cyclosporine, etretinate, methotrexate, or acitretin)	Grossman *et al.* [154]; Giannetti *et al.* [155]; de Jong *et al.* [156]; van de Kerkhof *et al.* [157]
Comparative studies of calcipotriol in combination with corticosteroids (betamethasone, clobetasone, or halobetasol)	Kragballe *et al.* [158]; Ruzicka *et al.* [159]; Ortonne [160]; Lebwohl *et al.* [161]
Calcipotriol in combination with betamethasone dipropionate (Daivobet®)	Kaufmann *et al.* [162]; Guenther *et al.* [163]; Douglas *et al.* [164]; Papp *et al.* [165]
Calcipotriol plus short-contact dithranol: A novel topical combination therapy for chronic plaque psoriasis	Monastirli *et al.* [166]

In contrast, a phase II study in hepatocellular carcinoma (HCC) showed encouraging results [130].

HCC is a primary malignant tumor of the liver, accounting for more than 300,000 cases per year [131]. The annual incidence is very high in Southeast Asia, with approximately 20 cases per 100,000 persons. The incidence in Western countries is considerably lower, but increasing. HCC has a very poor prognosis, and none of the drugs developed so far has resulted in an efficacious treatment of this disease.

The vitamin D receptor is expressed in normal liver tissue [132,133], with increased expression both in HCC [134] and in colorectal hepatic metastasis [135]. *In vitro* studies have shown that liver cancer cell lines also express the VDR and are responsive to the antiproliferative effects of $1,25(OH)_2D_3$ and analogs [136,137]. The effects of seocalcitol on HCC have been studied in C_3H/Sy mice, a strain with a very high incidence of spontaneous liver tumors. Only 4% of the mice treated with seocalcitol developed HCC, compared to 36% in the control group [88] (see Section IV.E.1).

These findings, coupled with the previously described metabolic stability and high affinity of seocalcitol for the liver, supported the decision to undertake the above mentioned phase II study with seocalcitol in patients with inoperable HCC [130]. Out of 33 patients evaluable for tumor response, two complete responses and 12 cases of stable disease have been reported from this study. Tumor tissue from one of the complete responders was tested for the presence of vitamin D receptors by Western blot and was found positive. At present, two phase III studies in patients with HCC are ongoing. One study is evaluating the efficacy of seocalcitol in prolonging survival of patients with HCC not amendable to curative treatment. Another study is evaluating the efficacy of seocalcitol in prolonging the time to relapse, following intended curative resection or percutaneous ablative treatment for HCC.

In conclusion, some of the novel, synthetic vitamin D analogs have shown clinical usefulness, and it is to be expected that their number will increase, together with our expanding knowledge of their chemistry and biology. Results from these studies will be made available at the NIH/Vitamin D workshop Meeting, November 2004, Bethesda, Maryland [167].

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Gemini: The 1,25-dihydroxy Vitamin D Analogs with Two Side-Chains

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I. INTRODUCTION

In a continuous search for analogs of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) (**1**) with improved biological profiles, the chiral center at carbon 20 became one of the focal points in structure-activity studies when it was shown that 20-epi-$1,25(OH)_2D_3$ (**2**) was a thousand times transcriptionally more active than the natural hormone. Subsequent mechanism investigations by Peleg and Norman [1,2], Moras [3], and Carlberg [4] indicated that **2**, once bound to the vitamin D receptor (VDR) ligand-binding domain produces the same agonist conformation as $1,25(OH)_2D_3$, but with an increased half-life and transcriptional activity. The transcriptional activities of $1,25(OH)_2D_3$ (ED_{50} $3.0 \pm 1.2 \times 10^{-9}$ M) and its 20-epi analog ($10.0 \pm 0.5 \times 10^{-12}$ M) were compared in ROS 17/2.8 cells transfected with a fusion gene containing the osteocalcin vitamin D response elements (VDRE) fused to the thymidine kinase promoter/growth hormone reporter gene.

Peleg and co-workers [2] tested the stability of **1** and **2** VDR complexes under non-equilibrium conditions, similar to those developed during prolonged incubation of cell cultures. They found that only 35% of the VDR binding sites remained occupied with **1** three hours after the excess of the ligand was removed from the treated VDR-transfected COS-1 cells. In the case of **2**, however, 80% of the VDR binding sites remained occupied three hours after the removal of its excess. This reduced dissociation rate contributes to a longer half-life of 20-epi-$1,25(OH)_2D_3$–VDR complex and to its increased transcriptional activity (see Fig. 1).

Since **1** or its 20-epi analog **2** occupy only 56% of the space in the ligand-binding VDR domain, we were intrigued by the possibility that a compound such as **3** with two side chains emanating at carbon 20 would accommodate one of its side chains according to $1,25(OH)_2D_3$ and the other according to 20-epi analog occupied spaces in the agonist conformation of the ligand-binding VDR domain, thus both contributing to the transcriptional activity.

II. SYNTHESIS OF GEMINI

The two-side-chain analog **3** generally referred to as Gemini, was synthesized as outlined in Fig. 2. The protected olefin **6** is easily accessible in high yield from the Inhoffen-Lythgoe diol via **4** and the iodide **5**. A double-ene reaction with ethyl propiolate produced the conjugated diester **7**. In this two-step process the mono-*ene* reaction product is formed rapidly at an early stage of the reaction. Compound **7** was hydrogenated with palladium on carbon catalyst, and the resulting **8** converted to the diztertiary alcohol **9** by a Grignard reaction [5].

The following steps in the synthesis are reminiscent of the protocol used for the synthesis of $1,25(OH)_2D_3$ [6]. Removal of the alcohol protecting group in **9**, oxidation, and protection of two tertiary hydroxyls produced the ketone **10**. The Lythgoe coupling with the precursor of
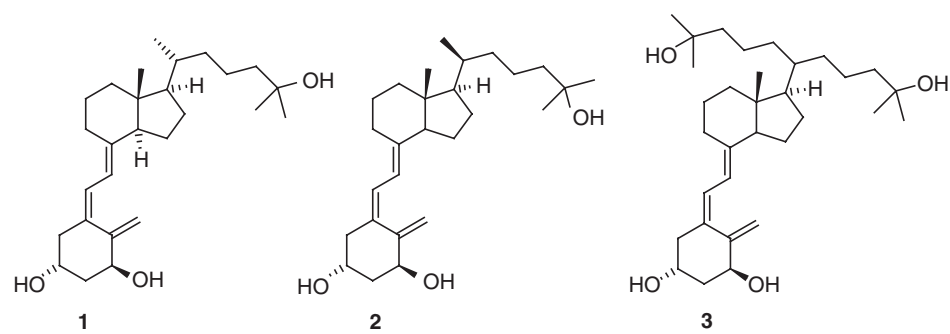
FIGURE 1
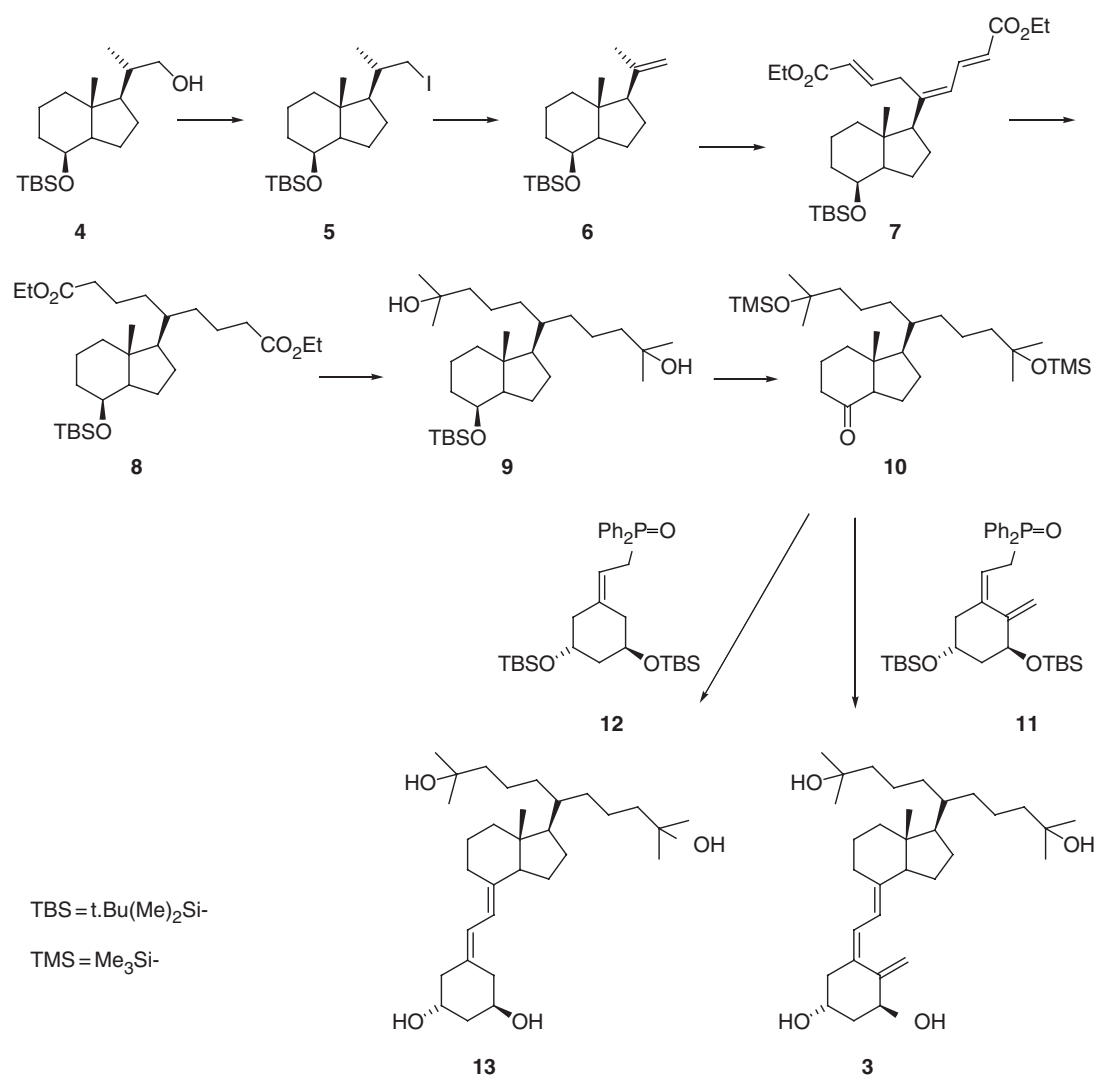

TBS = t.Bu(Me)₂Si-

TMS = Me₃Si-

FIGURE 2

the ring A **11** and deblocking of hydroxyl groups resulted in Gemini **3**. Using the 19-nor-ring A precursor **12** instead of **11** gave the 19-nor Gemini **13**.

III. 24R-HYDROXY GEMINI METABOLITE

Metabolic study of Gemini (**3**) and 19-nor-Gemini (**13**) in perfused rat kidney and bone cells indicated formation of a mono-24-hydroxy metabolite in each case, which were stable to further metabolism. Insertion of the 24R-hydroxy group in one of the side chains produces a stereogenic center at C-20, thus one of the two possible 20-epimeric metabolites can be formed in each case. Figure 3 displays the structures of 20-epimeric-24R-hydroxy Gemini and 19-nor-Gemini.

To determine the C-20 configuration of the 24R-monohydroxylated metabolites, the four compounds shown in Fig. 3 were needed for a HPLC comparison. They were obtained by synthesis starting from C-20-epimeric intermediates **23** and **24** (Fig. 4). Compounds **23** and **24** were prepared starting from previously shown **6**, the side chain of which was extended first by an *ene* reaction to give **18**, then by malonate alkylation to form **20**. Decarboxylation and the methyl

Grignard reaction produced the full side chain not bearing the 24R-hydroxy group. Hydroboration and separation completed the formation of **23** and **24** in a 2:3 ratio.

The primary alcohol **24** was converted to sulfone **27**, which was then alkylated with the known tosylate **28**, thus incorporating the required 24R hydroxy group. Reductive removal of the sulfone group and deblocking of hydroxyl groups produced the tetrol **32** representing the complete CD-ring side-chain portion of the target 20(S)-compounds **15** and **17**. The completion of their synthesis required temporary protection of the vicinal diol **32**, oxidation to **34**, replacement of the hydroxy blocking groups as in **36**, and the Lythgoe attachment of the A-rings **11** and **12** (Fig. 5).

Starting from the 20S-alcohol **23** (Fig. 4) the corresponding 20R-analogs **14** and **16** were prepared (see Fig. 6).

Metabolism products of Gemini (**3**) in the isolated perfused rat kidney were compared with those of **1**. The technique of rat kidney perfusion to study the metabolism of various vitamin D_3 analogs was previously described in several publications [7–9]. As previously reported, **1** is metabolized into calcitroic acid and more than 50% of substrate $1,25(OH)_2D_3$ had disappeared from the kidney perfusate due to its
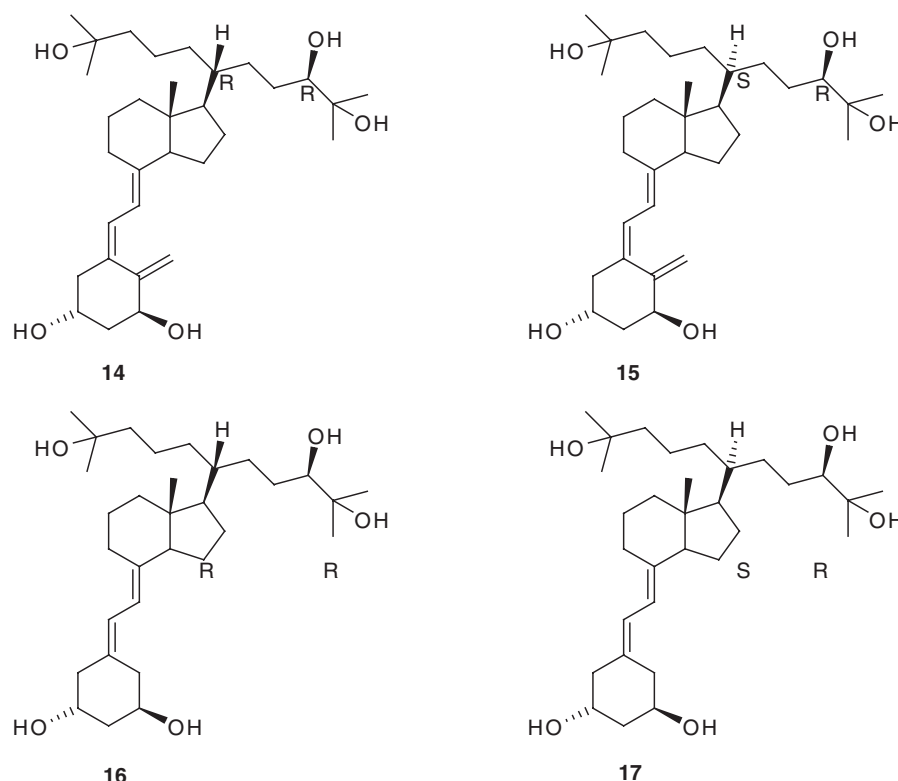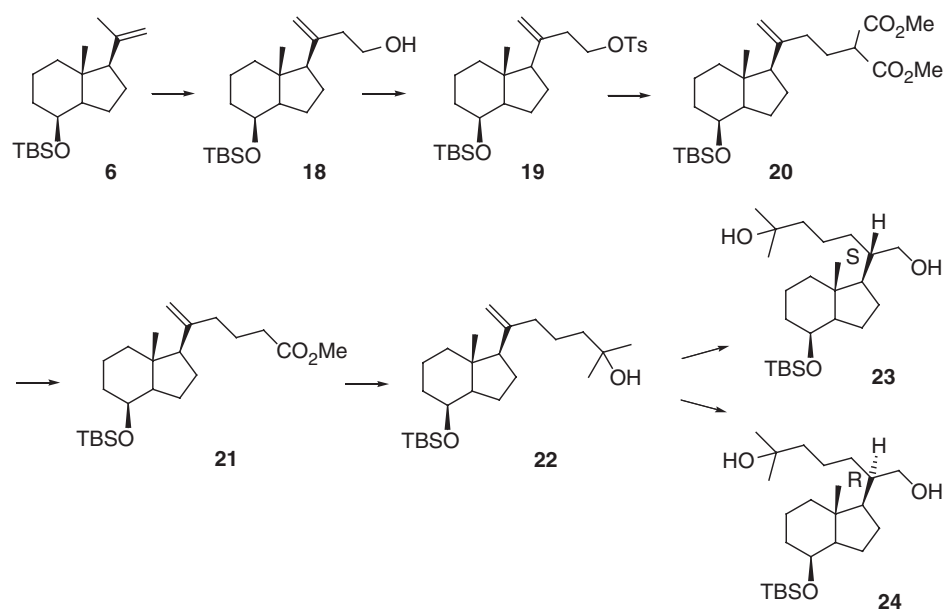

FIGURE 3

Figure 4

rapid metabolism through C-24-oxidation pathway. Unlike **1**, the Gemini analog resisted its metabolism through C-24 oxidation pathway and only about 20% of the substrate disappeared from the kidney perfusate. Through the analysis of the perfusate for daughter metabolites, we isolated only one polar metabolite. This metabolite was identified by mass-spectrometry and its sensitivity to periodate oxidation as the mono-24-hydroxylated metabolite of Gemini (**3**). Thus, it appears that Gemini is metabolized in rat kidney at a reduced rate when compared to **1**. Also, there appears to be a block to the further metabolism of 24(OH)-Gemini metabolite.

The studies were repeated using 19-nor Gemini **13**. Like the Gemini analog **3**, the 19-nor Gemini analog **13** is also metabolized in the rat kidney to a single polar metabolite and this metabolite was identified as the mono 24-hydroxylated metabolite of 19-nor Gemini. The same metabolite was also produced in the rat osteosarcoma cells (UMR 106). As shown in Fig. 7 of the HPLC comparison with the synthetic epimers **16** and **17**, the structure and stereochemistry of the 19-nor Gemini metabolite is 20R,24R (**16**).

IV. THE 23-YNE-26,27-HEXAFLUORO GEMINI ANALOGS

To prevent the 24R-hydroxylation of Gemini and increase its genomic activity, the introduction of C_{23}–C_{24} triple bond and 26,27-hexafluoro substitution were

investigated as shown in Fig. 7. The formaldehyde *ene* reaction of the olefin **22** (Fig. 4) gave the mixture of the *cis* and *trans* homoallylic alcohols **37**. Hydrogenation of **37** with Pt-on carbon catalyst gave the clean conversion to the desired **38** and **39** in nearly equal proportion, which were separated quantitatively by chromatography. The extension of the side chain of the epimer **39** led ultimately to the hexafluorohydroxy ketone **44**, the structure of which was established as 20(S) by an X-ray analysis. The attachments of the A-rings then produced the desired acetylenic hexafluoro Gemini **45** and its 19-nor analog **46**. Using the same pathway as described in Fig. 8 but starting the 20(R)-diol **38**, the corresponding 20(R)-hexafluoro analogs **47** and **48** were obtained.

The C-20 stereochemistry of the 24R-hydroxy Gemini analogs **14**, **15**, **16**, and **17** rests on the following established chemical connection shown in Fig. 8.

V. GEMINI ANALOGS AS RENIN INHIBITORS

As discussed in Chapter 52, 1,25-dihydroxyvitamin D_3 is an endocrine inhibitor of the renin-angiotensin system (RAS) *in vivo* [10]. The RAS plays an essential role in the regulation of blood pressure, volume, and electrolyte homeostasis. Overstimulation of the RAS is among the major causes for the development of hypertension. Therefore, components of the RAS have been important drug targets for the treatment of hypertension [11].
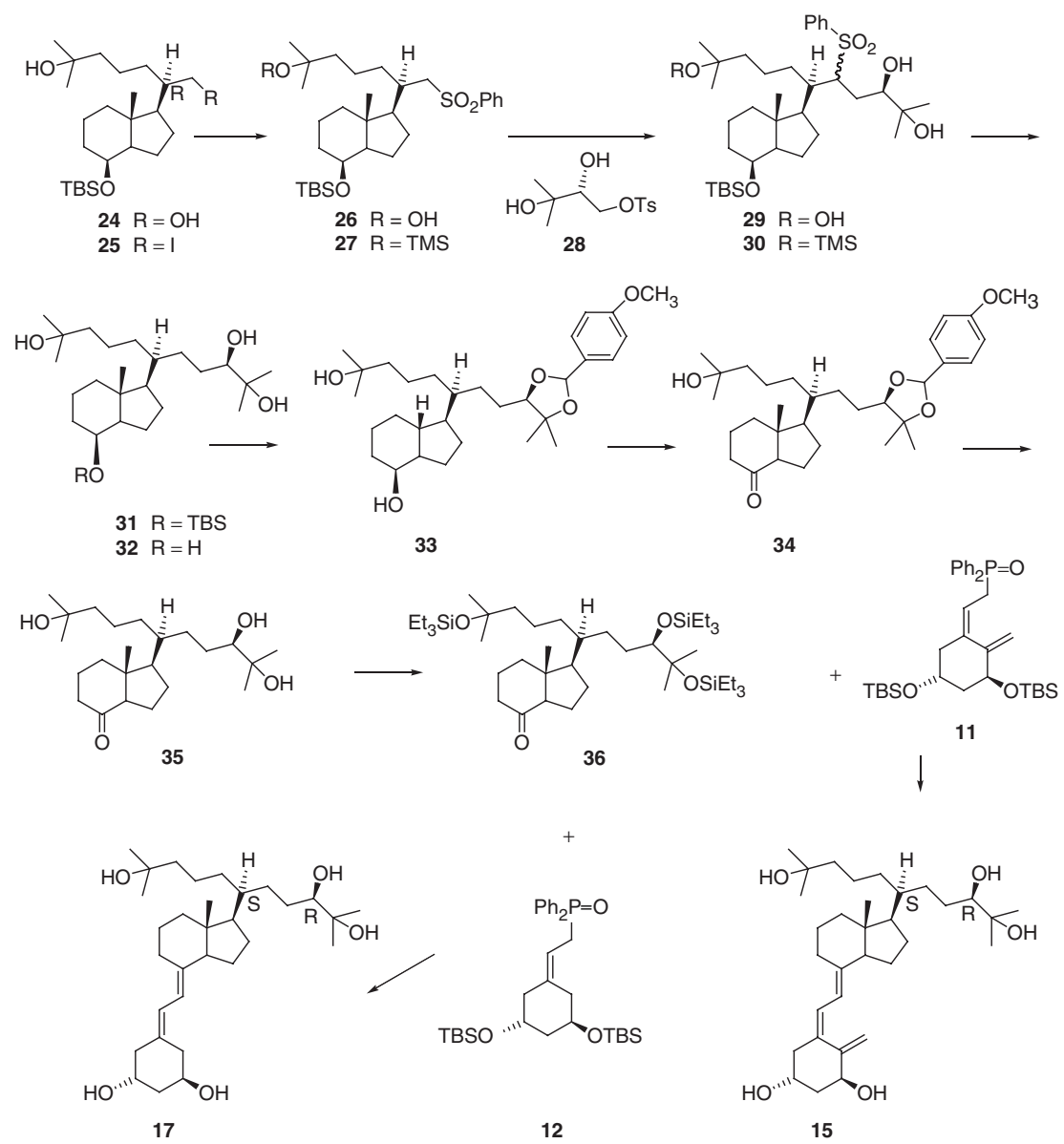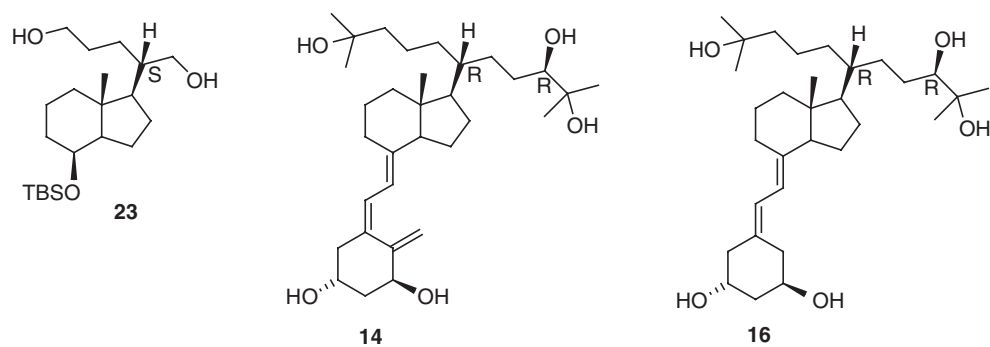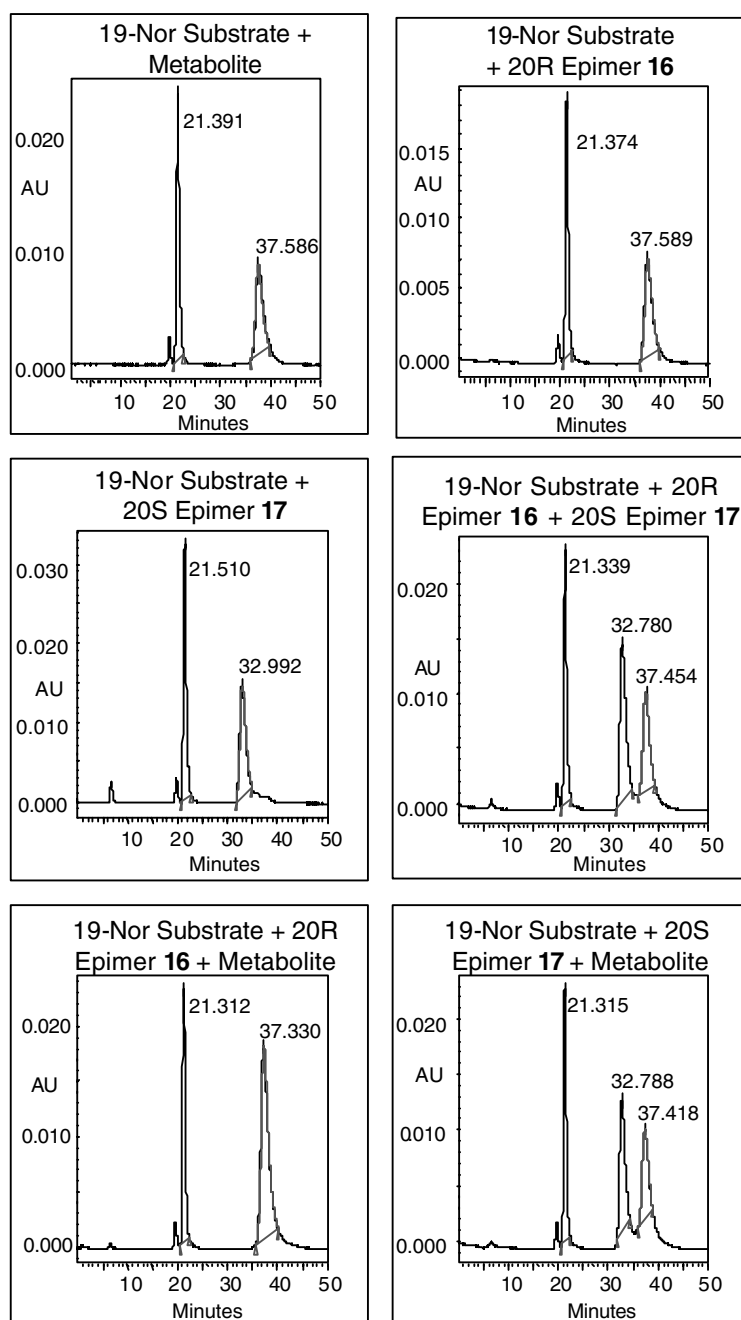
FIGURE 5

FIGURE 6

FIGURE 7 HPLC profiles of various combinations of mono-24-hydroxylated metabolite of 19-nor Gemini and the two synthetic standards (20R epimer **16** and 20S epimer **17**). HPLC analysis was performed using a straight phase HPLC system. Chromatographic conditions: Zorbax Sil column (9.4 mm × 25 cm; 15% 2-propanol in hexane, flow rate 2 ml/min.)

The renin-inhibiting activity of **1** suggests that low calcemic vitamin D analogs may potentially be used as therapeutic agents to control renin production and thus blood pressure.

The analogs of **1** were screened for the activity to inhibit renin production, using an *in vitro* cell culture system, followed by animal testing. The activity was determined by Northern blot and renin promoter luciferase reporter assays in As4.1 cells stably transfected with human VDR cDNA [12]. Interestingly, of 20 vitamin D analogs that have been screened so far, only gemini compounds display potency equal to or better than **1**, whereas the other single side-chain analogs have little inhibitory activity. Why the double-side chain Gemini compounds possess particularly potent inhibitory activity remains to be explored. Table I summarizes the
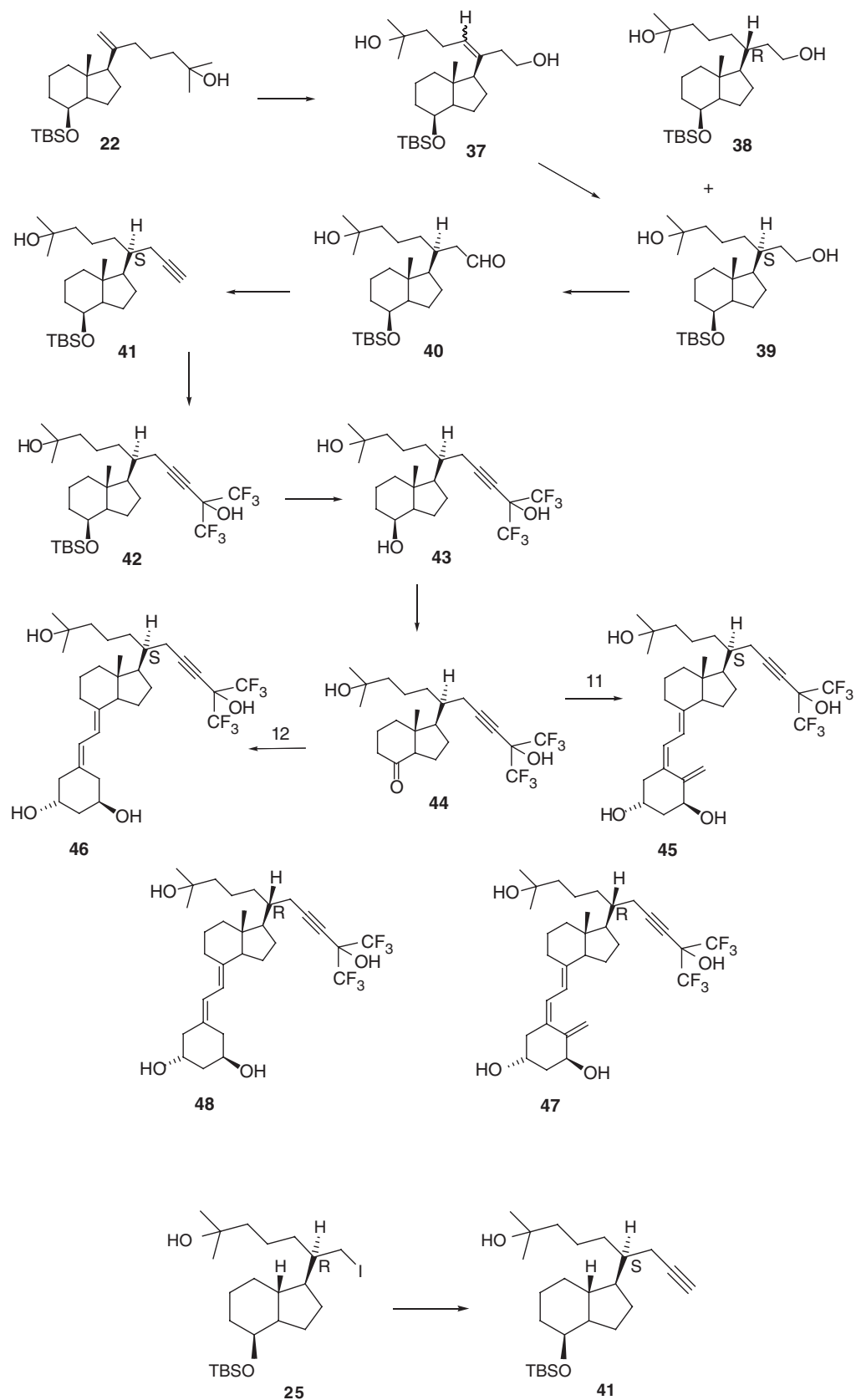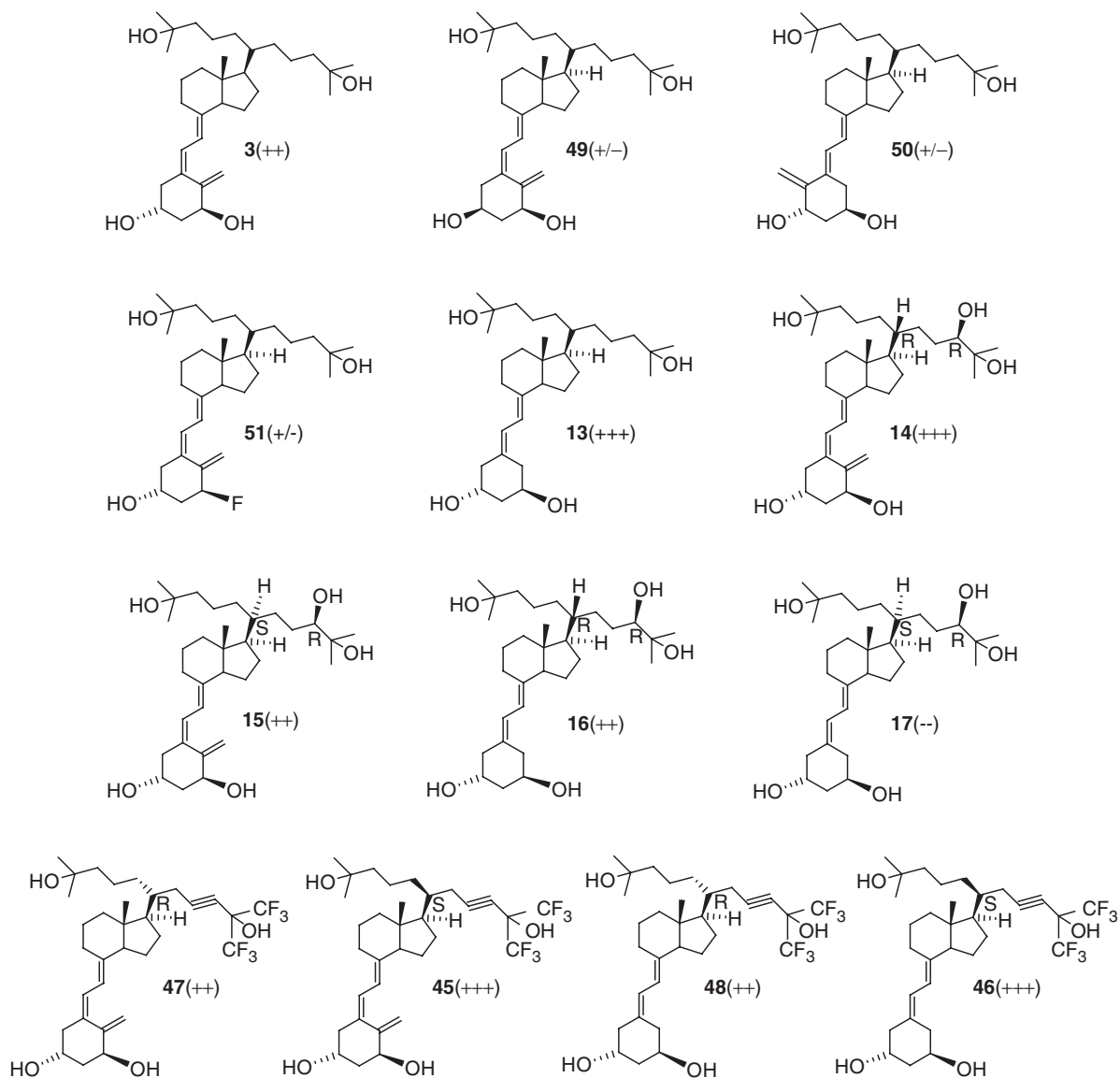
FIGURE 8

TABLE I

Relative Activity of Gemini Compounds to Inhibit Renin Expression in As.4.1 Cells

The inhibitory activity was determined by measuring the renin mRNA level with Northern bolt after treating As4.1-hVDR cells with each Gemini compound for 24 hours at 10^{-8}, 10^{-9}, and 10^{-10} M. The relative activity is based on that of 1,25-dydroxyvitamin D_3, which is arbitrarily set at (++).

relative activity of 13 Gemini compounds tested in cell cultures. The *in vivo* efficacy of the active Gemini compounds was further tested in mice. Of the eight active compounds listed in Table I, the 19-nor Gemini **13** shows the best efficacy to suppress renin expression *in vivo*. Daily intraperitoneal injection of this compound into normal mice for one week significantly inhibits renin mRNA expression in the kidney. Whether this or other Gemini analogs can indeed reduce blood pressure in high-renin hypertension requires further investigations. Given the fact that low calcemic vitamin D analogs have been approved for a number of clinical applications [13], Gemini compounds may offer a new source for the long-sought therapeutic renin inhibitors [14,15], which may potentially be developed into another class of anti-hypertensive drugs.

VI. INHIBITION OF ACUTE ALLOGRAFT REJECTION BY GEMINI ANALOGS

Acute allograft rejection is mediated by immunological mechanisms, with dendritic cells (DCs) and T-cells playing a major role. Current immunosuppressive treatments based on small molecules (cyclosporine A, tacrolimus, sirolimus, mycophenolate mofetil) or on biologicals (anti-CD3, anti-CD52, anti-IL2R) target mostly T-lymphocytes rather than antigen presenting cells (APCs). Drugs targeting both cell subsets would therefore represent a useful addition to the available immunosuppressive agents.

The immunomodulatory properties of **1** and its analogs suggest the clinical applicability of these hormones also in the treatment of allograft rejection, with the aim of facilitating tolerance induction and preventing chronic graft rejection. VDR ligands have pleiotropic immunoregulatory activities that are able to control allograft rejection. APCs and T-cells can be direct targets of the hormone, leading to the inhibition of pathogenic effector T-cells and enhancing the frequency of T-cells with suppressive properties, largely via induction of tolerogenic DCs.

These immunoregulatory activities, coupled with the absence of major side effects once calcemia is under control, have been translated into effective immunointervention in a variety of graft rejection models [16,17]. Induction of tolerance to allografts remains an unfulfilled goal in clinical transplantation, and VDR ligands could be part of a tolerogenic regimen designed to achieve this goal. VDR ligands have indeed gained widespread clinical application, notably in the treatment of secondary hyperparathyroidism and psoriasis, but hypercalcemia is a dose-limiting effect that prevents sustained systemic administration. To overcome this limitation, a number of $1,25(OH)_2D_3$ analogs, with a wider therapeutic window than $1,25(OH)_2D_3$ (**1**) itself, have been synthetized and shown effective in experimental models of autoimmune diseases and allograft rejection [18].

A. Immunomodulatory Properties of VDR Ligands in the Inhibition of Graft Rejection

APCs, and notably DCs, are key targets of VDR ligands, both *in vitro* and *in vivo*. $1,25(OH)_2D_3$ (**1**) and its analogs inhibit the differentiation and maturation of DCs [19], a critical APC in the induction of T-cell-mediated immune responses. These studies, performed either on monocyte-derived DCs from human peripheral blood or on bone-marrow derived mouse DCs, have consistently shown that *in vitro* treatment of DCs

with **1** and its analogs leads to down-regulated expression of the costimulatory molecules CD40, CD80, CD86 and to decreased IL-12 and enhanced IL-10 production, resulting in decreased T-cell activation. The abrogation of IL-12 production and the strongly enhanced production of IL-10 highlight the important functional effects of **1** and its analogs on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties.

The prevention of DC differentiation and maturation, as well as the modulation of their activation and survival leading to DCs with tolerogenic phenotype and function, and to T-cell hyporesponsiveness [20], certainly play an important role in the immunoregulatory activity of **1**. These effects are not limited to *in vitro* activity: **1**, and its analogs can also induce DCs with tolerogenic properties *in vivo*, as demonstrated in models of allograft rejection by oral administration directly to the recipient [21] or by adoptive transfer of *in vitro*-treated DCs [22]. Tolerogenic DCs induced by a short treatment with **1** are probably responsible for the capacity of this hormone to induce $CD4^+CD25^+$ regulatory T-cells that are able to mediate transplantation tolerance [21].

$1,25(OH)_2D_3$ (**1**) *in vivo* appears primarily to inhibit Th1-cells and, under appropriate conditions, may favor a deviation to the Th2 pathway. These effects could be, in part, a consequence of direct T-cell targeting by **1** and its analogs, but modulation of APC function by VDR ligands certainly plays an important role in shaping the development of T-cell responses. The capacity of VDR ligands to target APCs and T-cells is mediated by VDR expression in both cell types and by the presence of common targets in their signal transduction pathways, such as the nuclear factor NF-*k*B that is down-regulated in APCs [23] and in T-cells [24].

The immunoregulatory properties of **1** and its analogs have been demonstrated in different models of experimental organ transplantation, both acute and chronic [16]. $1,25(OH)_2D_3$ and its analogs can significantly prolong allograft survival in heart, kidney, liver, pancreatic islets, skin, and small bowel allografts. In general, these effects have been achieved at the maximum tolerated dose, without inducing hypercalcemia, the major side effect of treatment with VDR ligands. In most experimental models, the acute rejection has been further delayed by combining VDR ligands with a suboptimal dose of CsA or other immunosuppressive agents.

The induction of tolerogenic DCs by VDR ligands, which leads to an enhanced number of $CD4^+CD25^+$ regulatory T cells *in vivo* [21,25], is likely to play an important role in controlling graft rejection, both acute and chronic, and in favoring the establishment of

transplantation tolerance. A short treatment with **1** and mycophenolate mofetil, a selective inhibitor of T- and B-cell proliferation that also modulates APCs, induces tolerance to islet allografts associated with an increased frequency of CD4+CD25+ regulatory T-cells able to adoptively transfer transplantation tolerance [21].

Also the direct effects of VDR ligands on T-cells, in particular the inhibition of IL-2 and IFN-γ production, could play a role in inhibiting graft rejection [16]. **1** is believed to inhibit IL-2 secretion by impairing the formation of the transcription factor complex NF-AT and IFN-γ through interaction of the ligand-bound VDR complex with a VDRE in the promoter region of the cytokine. A combination of **1** and low-dose CsA inhibited the expression of IL-2 and IL-12, and increased significantly IL-10 expression levels in kidney allografts. Additional mechanisms could rely on the capacity of $1,25(OH)_2D_3$ to significantly reduce bioactive renal TGF-β1 by interacting with Smad proteins, important regulators of TGF-β signal transduction.

Based on the available evidence of a pro-tolerogenic effect and a reduced incidence of chronic rejection, VDR ligands could be added to standard immunosuppressive regimens in the treatment of allograft rejection. Additive and even synergistic effects have been observed between **1** or its analogs and immunosuppressive agents, in particular CsA, tacrolimus, and sirolimus [26]. These effects have been confirmed in models of graft rejection, making VDR ligands potentially interesting as dose-reducing agents for conventional immunosuppressive drugs in clinical transplantation.

Another positive feature of adding VDR ligands to standard immunosuppressive regimens is their protective effect on bone loss. A rapid bone loss is usually seen after organ transplantation and is enhanced by some immunosuppressive regimens, in particular those based on tacrolimus and steroids. Administration of **1** has been shown to prevent bone loss in transplanted patients, although standard prophylactic measures may not always be sufficient to prevent loss of bone mass, and $1,25(OH)_2D_3$ analogs with a wider therapeutic window also could serve this function. In addition, the $1,25(OH)_2D_3$ analog 22-oxa-$1,25(OH)_2D_3$ (OCT) has been shown to exert an anabolic effect on bone reconstruction by vascularized bone allografts in rats [27], indicating a specific advantage of VDR ligand administration in bone transplantation. In addition to avoiding bone loss, the use of VDR ligands to increase transplant survival does not appear to increase opportunistic infections, an important side effect induced by anti-rejection drugs, in particular calcineurin inhibitors and glucocorticoids.

B. Inhibition of Vascularized Heart Allograft Rejection by Treatment with the 19-nor Gemini Analog 13

Heterotopic cardiac transplants in mice were performed between C57BL/6 donors and BALB/c recipient mice as described by Corry [28]. To determine its capacity to inhibit acute allograft rejection in the vascularized heart transplantation model, 19-nor Gemini (**13**) was administered daily orally at a dose of 10 μg/kg/day starting the day before transplantation until day +1 followed by 3 μg/kg/day from day +2 until +30, while **1** was administered 3 d/week orally at a dose of 5 μg/kg/day starting the day before transplantation until rejection. Vehicle alone (Mygliol 812) was used as control. Administration of the Gemini analog **13** as monotherapy to mice receiving fully MHC-mismatched cardiac allografts resulted in a statistically significant prolongation of graft survival compared to administration of vehicle alone (mean survival 52 ± 29 vs. 9 ± 0.9 days, $p = 0.023$). Prolongation of graft survival was over three times more sustained following treatment with 19-nor gemini **13** compared to **1** (Table II). This marked inhibition of acute graft rejection was maintained in 2 of the 3 mice tested following treatment withdrawal and was achieved without inducing hypercalcemia after 15 or 20 administrations, whereas **1** induced a slight hypercalcemia. These results show the potent *in vivo* immunosuppressive effect of Gemini analog **13** in the context of an acute model of
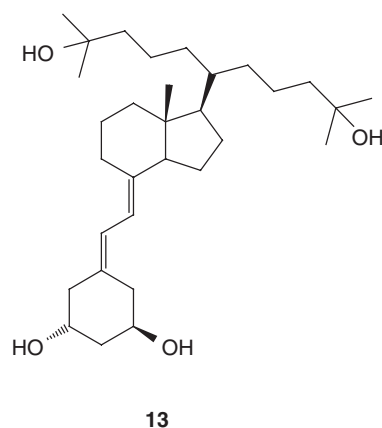
TABLE II   Delayed Acute Rejection Following VDR Ligand Administration

n	Treatment	Dose	Graft survival time	Mean	SD	P value	Ca mg/dl
6	vehicle	–	8, 8, 9, 9, 10, 10	9.00	0.9	–	8.8
6	$1,25(OH)_2D_3$ (**1**)	5[a]	12, 12, 14, 15, 16, 17	14.33	2.1	0.0022	10, 9 (15×)
3	19-nor Gemini (**13**)	10[b] – 3[c]	30, 41, 85	52.00	29.0	0.023	10 (15×); 10.5 (20×)

[a]Treatment per os (3×/w) every other day from d − 1 until d + 30.

[b]μg/kg po (d − 1,0, +1).

[c]μg/kg po (d + 2, until d + 30).

13

allotransplantation, and demonstrate that its efficacy is superior to that of **1**.

VII. RATIONALE FOR USING GEMINI ANALOGS TO TREAT COLON CANCER

It is recognized that $1,25(OH)_2D_3$ (**1**) is a potent inhibitor of cellular proliferation and inducer of maturation of a wide variety of cultured cancer cells, including breast cancer, colon cancer, prostate cancer, melanoma, lung cancer, and leukemia. Tanaka *et al.* [29] were the first to demonstrate that M-1 leukemic cells and HL-60 cells, which had VDR, were responsive to the antiproliferative activity of **1** and its analog 1α-hydroxyvitamin D_3 (1α-OH-D_3). They demonstrated *in vivo* that 1α-OH-D_3 was effective in treating M-1 leukemia in mice by prolonging the lives of mice that received the analog. Koeffler *et al.* [30] initiated human clinical trials with **1** in preleukemia patients and found that although there was an initial response, the patients often developed hypercalcemia and ultimately succumbed to their disease. It has been thought that cancer cells developed a resistance to **1** by either decreasing VDR, altering the $1,25(OH)_2D_3$-VDR-RXR interaction with transcriptional factors on the VDRE, and/or enhancing its catabolism by stimulating the 25-hydroxyvitamin D-24-hydroxylase [31].

The effectiveness of the antiproliferative activity of **1** and its analogs has been amply demonstrated for the treatment of the hyperproliferative skin disorder psoriasis [32]. Numerous analogs of **1** have been synthesized and evaluated for their antiproliferative and maturation activity, as well as their calcemic activity [33]. The goal for developing a potent vitamin D analog to treat some common cancers was to develop an analog that had marked antiproliferative activity without having any significant calcemic effects. Four studies have investigated

the *in vivo* response of vitamin D analogs in prostate cancer. The analogs were either slightly more potent or equally potent to $1,25(OH)_2D_3$ (**1**) and appeared to have less calcemic action than **1**. In a Phase I trial, patients with advanced hormone refractory prostate cancer received 1α-hydroxyvitamin D_2 [34]. Twenty-five percent of the patients achieved stabilization of their disease for six months with the main toxicity being hypercalcemia and renal insufficiency. Another strategy is to use **1** in combination with chemotherapy. The combination of a weekly oral high dose (0.5 µg/kg) of **1** and weekly docetaxel was shown to be well tolerated and effective in achieving a significant decrease in prostatic specific antigen in 30 of 37 metastatic androgen-independent prostate cancer patients [35].

A. Antiproliferative Activity of $1,25(OH)_2D_3$ and Its Analogs on Cultured Human and Rodent Colon Cancer Cells

Zhao and Feldman [36] demonstrated that $1,25$-$(OH)_2D_3$ (**1**) inhibited the proliferation of HT-29 human colon cancer cells in culture and induced them to terminally differentiate. This effect appeared to be directly related to the abundance of the VDR in the cells. Tananka *et al*. [37] reported in severe combined immunodeficient mice that a $1,25(OH)_2D_3$ analog, 22(S)-24-homo-hexafluoro-1,22,25-trihydroxyvitamin D_3, was effective in decreasing the invasion of human HT-29 colon cancer cells in the renal capsule, compared to placebo controlled animals. Serum calcium concentrations and body weights of the treated mice were similar in both groups demonstrating that the vitamin D analog most likely had a direct effect on inhibiting colon cancer cell growth.

We have grown both human and a mouse colon cancer cell lines in culture and found that **1** inhibited ^{3}H-thymidine incorporation in both Caco-2 and MC-26 cells (Fig. 9A, B). Cellular extracts of the MC-26 cells were obtained, and the RNA was extracted. Quantitative RT-PCR analyses revealed the presence of the VDR.

We have established an *in vivo* mouse colon cancer model by injecting 10,000 MC-26 cells subcutaneously in the dorsal posterior back of Balb-c mice that were six to eight weeks of age. Two days after tumor implantation, the mice were divided into three groups: control vehicle, $1,25(OH)_2D_3$ (0.1 µg/kg), 1,25-dihydroxy-(20S)-21-(3-methyl-3-hydroxy-4-butyl)-23-yne-26,27-hexafluoro-19-nor-vitamin D_3 (**46**) 1.0 µg/kg. The mice were weighed three times a week and their tumors measured every day (see Fig. 9). After 33 days, there was a marked increase in tumor volume in the mice that received placebo compared to
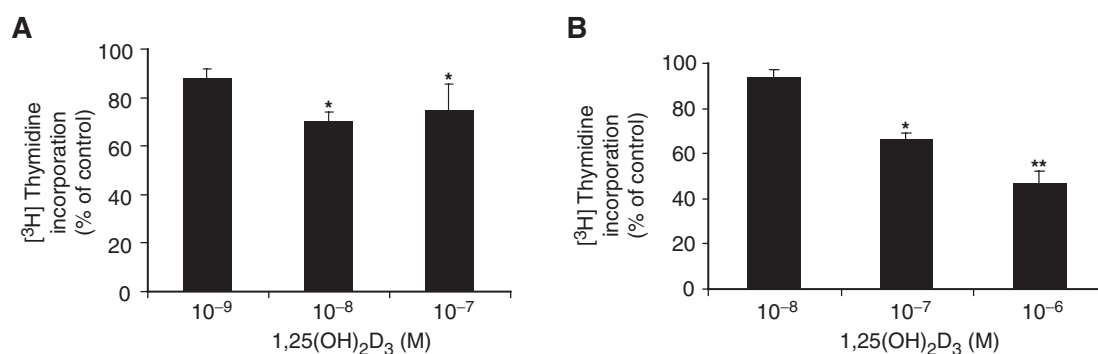
FIGURE 9   (A) Human colon cancer. Effect of $1,25(OH)_2D_3$ on ^3H-thymidine incorporation in a human (Caco-2). Results are means $\pm$ SEM $^*p < 0.005$. (B) MC-26 mouse colon cancer. Effect of $1,25(OH)_2D_3$ on the ^3H-thymidine incorporation in a mouse cancer cell line (MC-26). Results are means $\pm$ SEM. $^*p = 0.0003$ $^{**}p < 0.0001$.

the mice that received either **1** or **46**. After sacrificing the animals, the extent of tumor invasion was evaluated. 50% of the mice receiving placebo and 33% of the mice receiving $1,25(OH)_2D_3$ displayed muscle invasion by the tumor, whereas the group that received Gemini analog **46** showed no tumor extension into the adjacent musculature. At the end of the experiment, on day 33, the average tumor volume of the mice treated with placebo vehicle, **1** and **46** were 1,385 mm³, 692 mm³, and 352 mm³, respectively (see Fig. 10). Thus, the Gemini analog **46** reduced tumor volume by more than 75%. None of the mice that received the **46** analog died, whereas 35 and 30% of the mice receiving vehicle and **1** died at the end of the 33 days of the study (see Fig. 11). An evaluation of the serum calciums revealed a significant elevation in the animals treated with **1**, whereas the Gemini analog treated animals showed no evidence of hypercalcemia (Spina,

Tangpricha, and Holick, unpublished results) or body weight loss.

Therefore, these data suggest that the Gemini vitamin D analog **46** not only markedly decreased metastatic colon cancer activity, but it also prolonged life and caused no untoward consequences on the calcium metabolism in the mice. This study provides a promising new approach for treating colon cancer with this novel vitamin D analog.

In summary, a new class of 1,25-dihydroxy vitamin D receptor ligands, the two side-chain Gemini analogs are being investigated as the potential drug candidates for treatment of bone diseases, hypertension, acute allograft rejection, and colon cancer. Promising results have been obtained in the meaningful *in vivo* models for these indications. The methods for the synthesis of Gemini have been illustrated that allow their large-scale preparation.
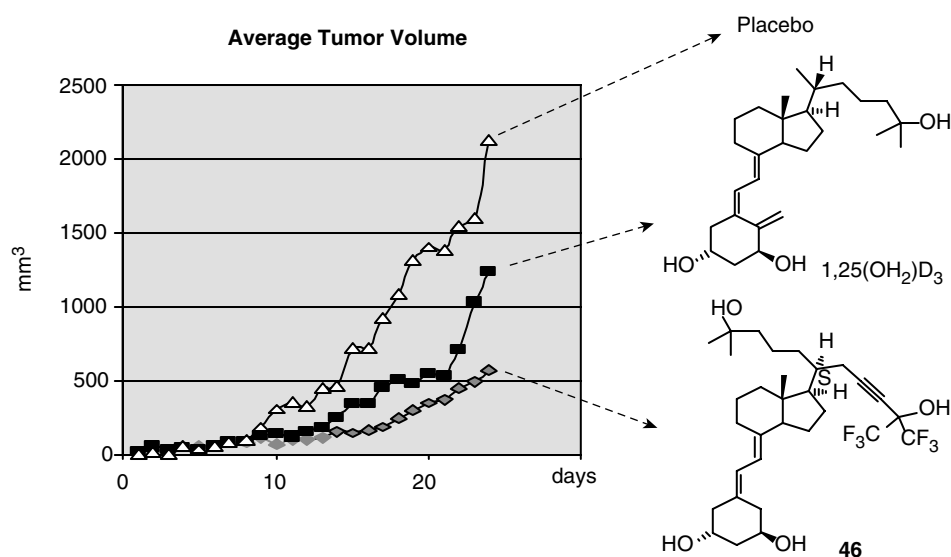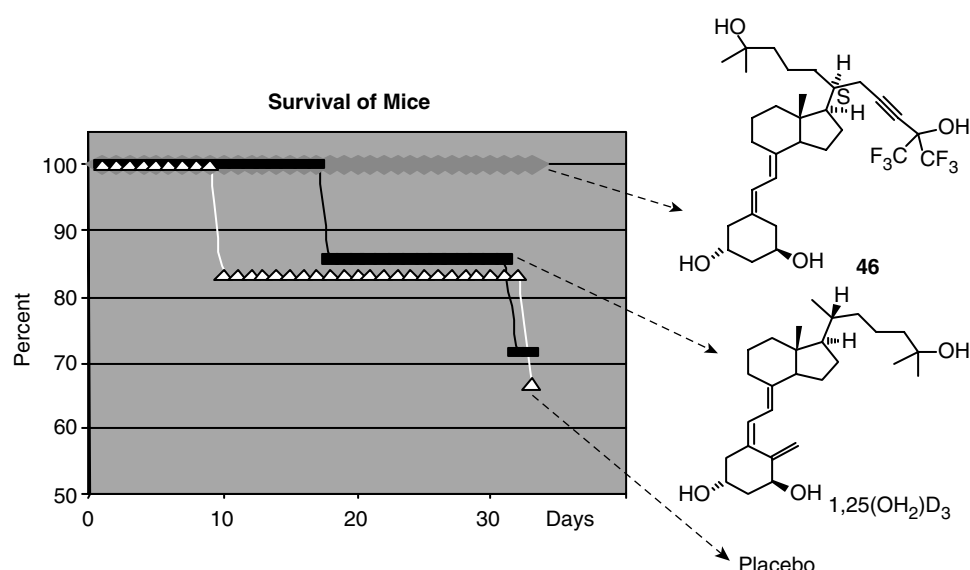

FIGURE 10

FIGURE 11

TABLE III   Calcium Levels (mg/dL)

Gemini (46)		1,25(OH)$_2$D$_3$		Control	
A1:1	8.4	B1:1	7.2	C1:1	8.4
A1:2	7.5	B1:2	12	C1:2	9.6
A1:3	8.4	B1:3	11.4	C1:3	9
A1:4	9.2	B2:1	7.8	C2:1	8.8
A2:1	6.8	B2:2	9.6	C2:2	10.8
A2:2	8.2	**Average**	**9.6**	**Average**	**9.32**
Average	**8.1**				

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Development of OCT and ED-71

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I. INTRODUCTION TO OCT AND ED-71

The active vitamin D_3, $1\alpha,25$-dihydroxyvitamin D_3 [calcitriol, $1,25(OH)_2D_3$], is now well-recognized as a potent regulator of cell proliferation and differentiation processes in addition to possessing regulatory effects on calcium and phosphorous metabolism [1]. Various analogs of $1,25(OH)_2D_3$ have been synthesized to separate differentiation-induction and antiproliferation activities from calcemic activity with the aim of obtaining useful analogs for the medical treatment of psoriasis vulgaris, cancer, *etc*., without manifestation of hypercalcemia [2]. $1\alpha,25$-Dihydroxy-22-oxavitamin D_3 (maxacalcitol, 22-oxacalcitriol, OCT) was obtained from such modification studies of the $1,25(OH)_2D_3$ side chain [3–11] (Fig. 1) and OCT has been shown to be highly effective in stimulating monocytic differentiation of human promyelocytic leukemic HL-60 cells, although OCT is less calcemic than $1,25(OH)_2D_3$ [12]. It is well known that $1,25(OH)_2D_3$ binds to the circulating vitamin D transport carrier, vitamin D–binding protein (DBP), whereby it is carried to various tissues that express vitamin D receptors (VDR) and taken up into the cell nucleus to exert its effects [13]. The basic activity of OCT as a vitamin D_3 analog is largely characterized by its weaker affinity for DBP and VDR, which is approximately 1/580 and 1/8, respectively, than that of $1,25(OH)_2D_3$ [14–15]. OCT in blood also has been shown to bind to low density lipoproteins (LDL) as well as to DBP, so that it is incorporated into cells or the nucleus via VDR or LDL receptors of the target organs [16].

There is also an intense interest in obtaining analogs more potent than $1,25(OH)_2D_3$ or 1α-hydroxyvitamin D_3 (alfacalcidol, $1\alpha OHD_3$), a clinically important prodrug of $1,25(OH)_2D_3$, in terms of regulatory effects on calcium and phosphorous metabolism, with the aim of treating bone diseases such as osteoporosis. $1\alpha,25$-Dihydroxy-2β-(3-hydroxypropoxy)vitamin D_3 (2β-(3-hydroxypropoxy)-calcitriol, ED-71) (Fig. 1) was obtained in our modification studies of the A-ring

of $1,25(OH)_2D_3$ [17–21]. ED-71 is characterized by (i) a hydroxypropoxy substituent at the 2β-position; (ii) a relatively long plasma half-life arising from its strong affinity for DBP which is approximately two times more potent than $1,25(OH)_2D_3$ [22]; (iii) its potential therapeutic effects on bone [23].

In the previous edition of this book, we described the primary characteristics of OCT and ED-71, which were originally recognized during the course of our exploratory research of these analogs [24]. Since then, OCT has been launched in Japan in 2000 as an injection for the treatment of secondary hyperparathyroidism associated with renal insufficiency and, in 2001, as an ointment for treating the skin disease, psoriasis vulgaris. Clinical trials of ED-71 as a promising candidate for the treatment of osteoporosis have been conducted also in Japan. This chapter describes updated information concerning the development of OCT for secondary hyperparathyroidism and psoriasis vulgaris and ED-71 for osteoporosis. A practical synthesis of OCT for industrial scale production along with a convergent, highly versatile synthesis of ED-71 are also discussed below.

II. DEVELOPMENT OF OCT FOR SECONDARY HYPERPARATHYROIDISM AND PSORIASIS VULGARIS

A. Practical Synthesis of OCT for Large Scale Production

During the development of OCT, a practical synthesis was needed for industrial scale production. An important objective in the synthesis of OCT was the facile introduction of the side chain, which is characterized by the γ-hydroxy ether linkage. Typically, this can be accomplished by the Williamson ether synthesis which involves the reaction between alkyl halides and alkoxides. While this is a well-known and general method, yields of such reactions are not always
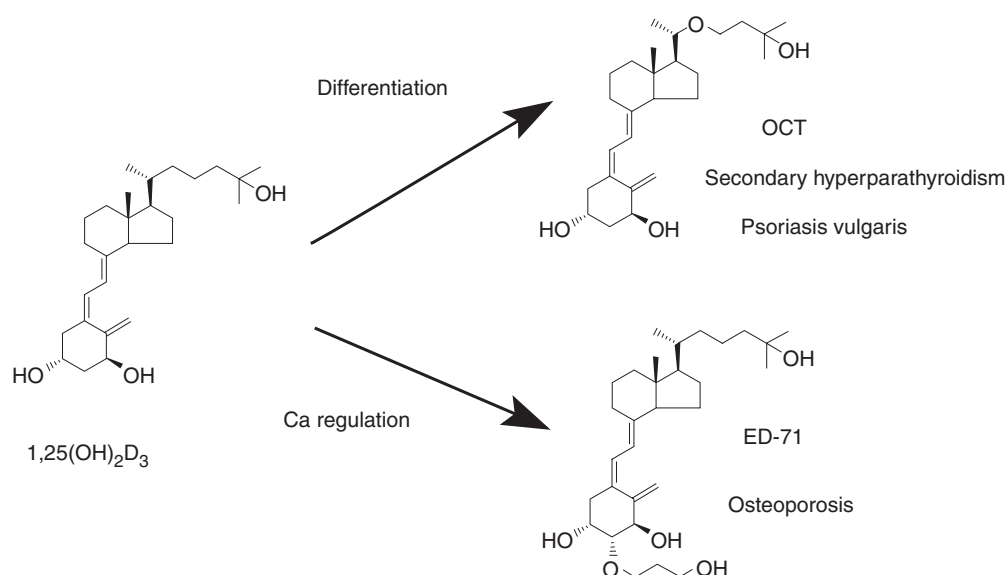
Figure 1   Chemical structure and therapeutic indications of OCT and ED-71.

satisfactory, particularly in sterically-hindered steroidal cases. Therefore, we investigated the practical introduction of the γ-hydroxy ether moiety of OCT by employing a two-step Michael and nucleophilic addition sequence.

In the total synthesis of OCT, we adopted an 11-step sequence as shown in Fig. 2. Dehydroepiandrosterone (DEA) was used as the starting material, which was converted to the 20(S)-alcohol via microbiological 1α-hydroxylation, silylation, 5,7-diene formation, Wittig olefination, and hydroboration. Alkylation of

the 20(S)-alcohol was accomplished via a conjugate addition reaction of N,N-dimethylacrylamide as the Michael acceptor in the presence of sodium hydride. Subsequent nucleophilic addition to the amide moiety with methyl magnesium chloride in the presence of cerium chloride gave the γ-hydroxy ether side chain of OCT in satisfactory yield. Desilylation, irradiation, and thermal isomerization produced OCT (Fig. 2) [25]. An improved one-pot synthesis of the γ-hydroxy side chain unit of OCT has been elucidated for industrial scale production.
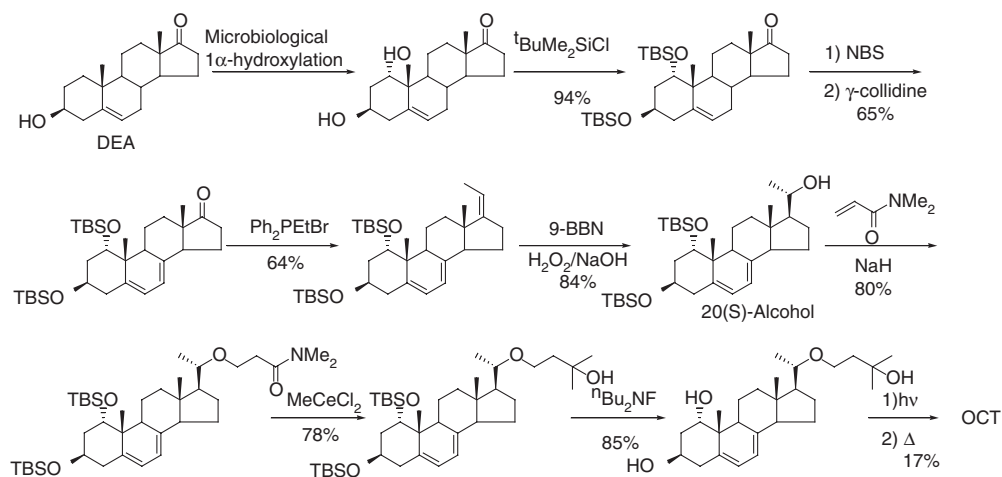

Figure 2   Practical synthesis of OCT for large scale production.

B. Secondary Hyperparathyroidism

1. PRECLINICAL RESULTS

a. Effects on Parathyroid Hormone   The inhibitory effect of $1,25(OH)_2D_3$ on parathyroid hormone (PTH) synthesis and secretion has been clearly elucidated (see Chapter 30). It has been reported that $1,25(OH)_2D_3$ blocks transcription via the negative vitamin D responsive region in the control region of the PTH gene [26] (see Chapter 30). OCT is also thought to exert its inhibitory action on PTH synthesis through the same mechanism as $1,25(OH)_2D_3$.

An *in vitro* study comparing the effects of OCT to $1,25(OH)_2D_3$ was performed using human hyperplastic parathyroid tissue (obtained during surgery for advanced renal hyperparathyroidism) and normal bovine parathyroid glands. OCT suppressed PTH secretion in both nodular hyperplastic parathyroid tissue and normal bovine tissue in a dose-dependent manner, analogs to $1,25(OH)_2D_3$ [27] (Fig. 3). The additive effect of cellular calcium levels on suppression of PTH secretion by OCT and $1,25(OH)_2D_3$ was

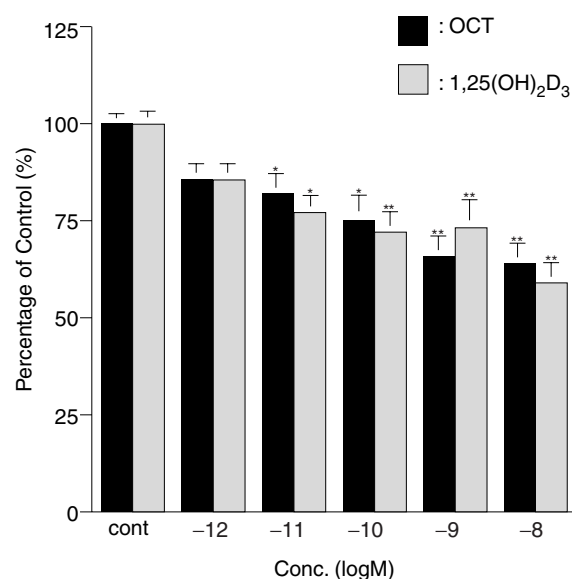

FIGURE 3   Effect of OCT or $1,25(OH)_2D_3$ on PTH secretion in primary cultures of parathyroid gland cells from patients with secondary hyperparathyroidism associated with chronic renal failure. Primary cultures of cells (calcium concentration in medium: 2 nmol/L) were incubated with added OCT or $1,25(OH)_2D_3$ (10^{-12} to 10^{-8} mol/L). After 48 hours of incubation the culture supernatant was removed and fresh medium added, followed by re-incubation for 2 hours. The amount of PTH secreted in 2 hours was determined by RIA (HS-PTH), and shown as percent of drug-free control. Data represent the mean ±S.D. (same hereunder; n = 22). Dunnett's test: control group vs. each treated group *p < 0.01; **p < 0.001; ■: OCT; □: $1,25(OH)_2D_3$.

also demonstrated. These results indicate that OCT suppresses PTH secretion, not only in normal parathyroid cells but also in hyperplastic parathyroid cells in much the same manner as $1,25(OH)_2D_3$.

An early study to examine the effect on parathyroid PTH gene expression in normal rats showed that OCT reduced parathyroid pre-pro PTH mRNA expression in 48 hours post-injection as also did $1,25(OH)_2D_3$ [28]. When assessed in a 5/6-nephrectomized rat model of chronic renal failure, intermittent administration of OCT lowered the circulating PTH level without causing an elevation in serum calcium concentration [29–31] (Fig. 4). Northern blot analysis of parathyroid pre-pro PTH mRNA expression in these same rats revealed significant inhibition of pre-pro PTH mRNA expression by OCT as well as by $1,25(OH)_2D_3$. OCT was thus considered to suppress PTH synthesis and secretion via inhibition of PTH gene transcription as seen in the case of $1,25(OH)_2D_3$ [29] (Fig. 5).

In the dog model of chronic renal failure, OCT treatment produced a significant decrease in levels of circulating serum PTH at week 5 of treatment, and the PTH level remained reduced by 80% consistently thereafter throughout the treatment period. Serum calcium concentrations rose significantly at week 13 of OCT administration compared to pretreatment levels, although this calcemic effect was inconspicuous [32].

For effects on calcium levels in the intestine, which is of general clinical concern in the use of vitamin D analogs, a comparative assessment of OCT was carried out in comparison to $1,25(OH)_2D_3$ in vitamin D–deficient rats. Calcium binding protein (Calbindin) D_{9K} gene expression was increased with both OCT and $1,25(OH)_2D_3$ until 16 hours after dosing but disappeared rapidly in the case of OCT, unlike $1,25(OH)_2D_3$ [33]. When assessed using the inverted gut sac technique, $1,25(OH)_2D_3$ proved to induce an active intestinal calcium transport that was characterized by a first phase sharp increase from 1 to 6 hours after administration and a second phase continuous rise from 12 hours post-dose onwards. Unlike the response to $1,25(OH)_2D_3$, the active intestinal calcium transport induced by OCT was practically nonexistent in the second phase. Increase in calcium absorption from the intestine associated with the use of OCT is thus considered less lasting as compared to $1,25(OH)_2D_3$ [33].

b. Effects on Bone   The slowly progressive renal failure rat has been used as an established animal model that is created through a single injection of glycopeptide isolated from rat renal cortical tissue [34]. In this model, OCT produced a significant decrease in serum PTH level without causing elevation of serum calcium. A histopathological evaluation of the effects of OCT on parathyroid and bone tissues in these
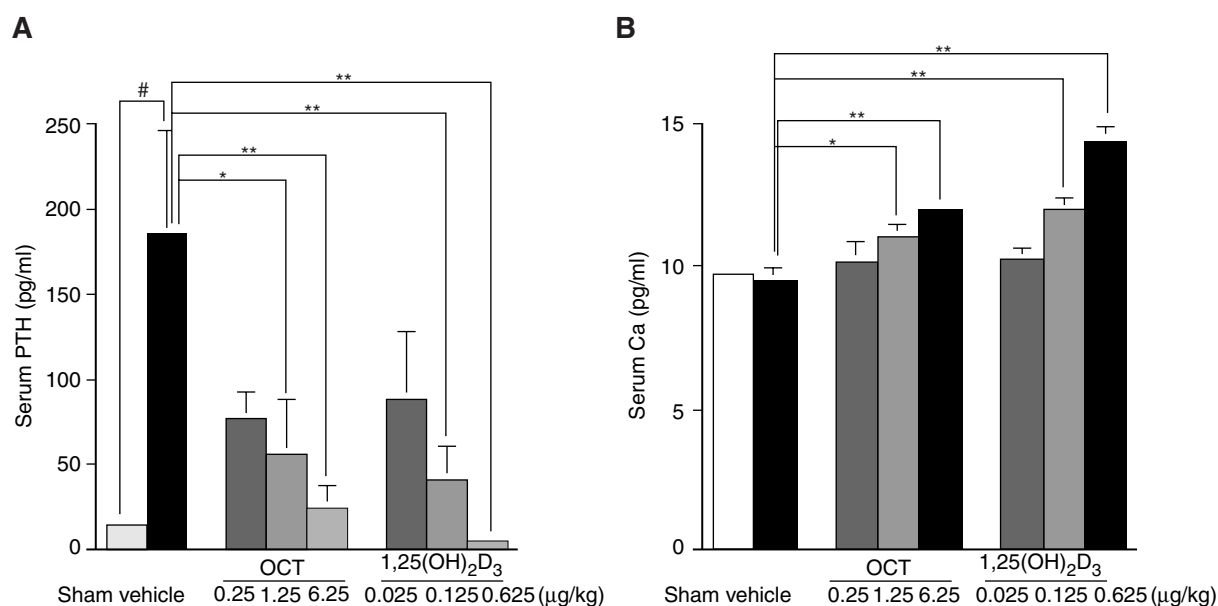
A

B

FIGURE 4   Effects of intermittent intravenous administration of OCT on serum PTH and calcium (Ca) levels in renal failure rats. 5/6-Nephrectomized rats were injected i.v. with OCT or $1,25(OH)_2D_3$ at various dose levels three times weekly for 15 days. Grouped t-test: sham-operated group vs. diseased control #$p < 0.05$. Dunnett's test: diseased control vs. each treated group *$p < 0.05$; **$p < 0.01$.
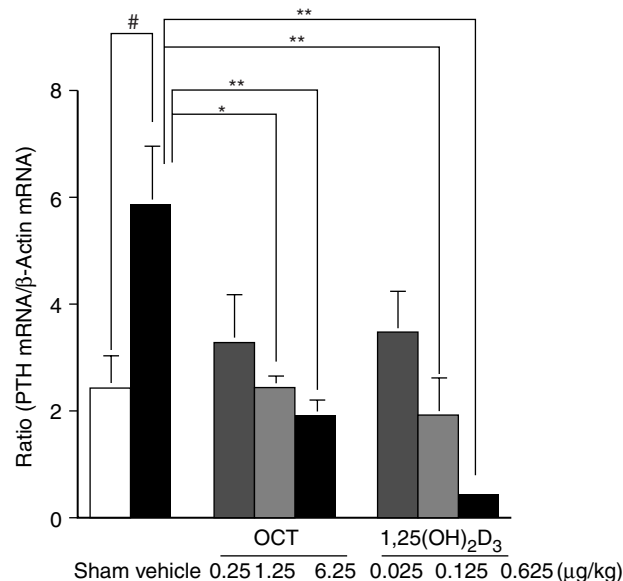

FIGURE 5   Effects of OCT and $1,25(OH)_2D_3$ on pre-pro PTH mRNA expression in renal failure rats. 5/6-Nephrectomized rats were injected i.v. with OCT or $1,25(OH)_2D_3$ at various dose levels three times weekly for 15 days. Parathyroids were excised 24 hours after the last dose and analyzed by Northern blot technique for pre-pro PTH mRNA expression, which is presented as its quotient divided by β-actin mRNA expression. Grouped t-test: sham-operated group vs. diseased control #$p < 0.05$. Dunnett's test: diseased control vs. each treated group *$p < 0.05$; **$p < 0.01$.

animals disclosed parathyroid hyperplasia with chief cell proliferation in virtually all rats of a diseased control group. In contrast, in the OCT-treated groups, hyperplasia was significantly reduced. Treatment with OCT reduced the incidence of ostitis fibrosa of the tibia and lumbar vertebrae in the OCT-treated groups (37.5–57.1%), compared to the diseased control group (66.7%) [35].

A further evaluation of the effects of OCT with respect to bone formation, bone resorption, and relevant dynamic parameters was performed using the third lumbar vertebra. The data revealed significant increases in bone formation rate, mineral apposition rate, mineralized surface and tetracycline double-labeled area, increases in number and area of osteoclasts, and a significant increase in bone resorption rate in the diseased control group. The significance of these histomorphomery parameters is discussed in Chapter 50. In OCT-treated groups, enhanced bone formation rate, mineral apposition rate, and mineralized surface areas and bone formation in tetracycline double-labeled areas were significantly reduced. There was a significant decrease in the increased osteoclast number and area, indicating a potential efficacy of OCT for high-turnover bones. These effects suggest that OCT might ameliorate osseous lesions in secondary hyperparathyroidism [35] (Table I).

Table I   Bone Histomorphometry in the Lumbar Vertebra at the End of the OCT Treatment

Parameters	GN			
	Control	Vehicle	OCT (0.03μg/kg)	OCT (0.15μg/kg)
Bone formation parameters				
MS/BS %	16.6 + 0.6	27.6 + 3.2	18.0 + 2.8	13.4 + 2.1[b]
BFS/BS μm³/μm²/year	39.8 + 2.3	116.3 + 14.0[a]	67.6 + 13.9[b]	42.2 + 7.9[b]
MAR mm/day	0.66 + 0.03	1.14 + 0.8[a]	1.00 + 0.05	0.83 + 0.07[b]
Bone resorption parameters				
ES/BS %	21.4 + 0.5	33.0 + 2.1[a]	28.2 + 2.2	23.5 + 1.5[b]
Rs.R μm³/μm²/year	0.61 + 0.00	1.67 + 0.29[a]	1.06 + 0.34	0.76 + 0.19
N.Oc/BS/mm	0.65 + 0.12	1.36 + 0.24	0.70 + 0.08	0.67 + 0.2[b]

Abbreviations are: MS/BS, mineralizing surface; BFS/BS, bone formation rate; MAR, mineral apposition rate; ES/BS, eroded surface; Rs.R, bone resorption rate; N.Oc/BS/mm, osteoclast number.

[a] $p < 0.05$ vs. Control group.

[b] $p < 0.05$ vs. Vehicle group.

The effect of OCT on bone lesions was investigated in a dog model with chronic renal failure to clarify the effect of long-term administration of OCT on bone metabolism. Groups of renal failure dogs received either vehicle alone or OCT for one year and were examined for effects on bone changes in secondary hyperparathyroidism. Pre- and post-treatment bone biopsies were performed, and the results showed significant increases in abnormal fibrous osteoid area (Wo.OS/BS, %) and fibrous tissue area (Fb.S/BS, %) in the bones of vehicle-treated dogs with progression to secondary hyperparathyroidism. Trabecular unit activation frequency (Ac.f) and circulating PTH were also significantly increased. In dogs given OCT, in contrast, Wo.OS/BS(%) was found significantly reduced as compared to the diseased control group; hence, a delay in progression of bone changes. There was no significant intergroup difference in Ac.f changes. These studies suggest, therefore, that OCT might retard the progression of bone changes in secondary hyperparathyroidism without inducing low bone turnover [36].

An ensuing, continued eight-month cross-over study demonstrated a significant decrease in Wo.OS/BS(%) in the OCT-treated dogs as compared to pre-treatment values in dogs assigned to the diseased control group whose initial Wo.OS/BS(%) and Fb.S/BS(%) values were high. Again, there was no conspicuous difference between pre- and post-treatment Ac.f values. In the animals that had initially been treated with OCT, serum PTH increased following the discontinuation of OCT administration, but this was not accompanied by any appreciable change in bone lesions; namely, the corrective effect of OCT on bones lasted even after the withdrawal of OCT [36].

The efficacy of OCT for improving bone tissues in clinical settings was presumed from the demonstrated ameliorative effects of OCT on bone changes in secondary hyperparathyroidism and also in bone remodeling animals. Furthermore, the marked improvement of ostitis fibrosa parameters and the very modest associated changes in bone turnover parameters indicated the possibility that OCT may become a therapeutic agent with merit, having a lower risk of low turnover bones which is of clinical concern for the use of $1,25(OH)_2D_3$.

2. Clinical Results with Secondary Hyperparathyroidism Patients

Results of the clinical studies conducted in Japan from 1992 to 1997 have raised the expectation that intravenous OCT administration may be an effective treatment for secondary hyperparathyroidism. Intravenous OCT therapy was shown to afford marked clinical efficacy even for severe secondary hyperparathyroidism through a mechanism whereby intravenously administered OCT directly inhibits PTH synthesis and secretion. The drug also can be administered to patients whose serum calcium is somewhat elevated prior to treatment, resulting in a satisfactory therapeutic response (see Chapter 76 for discussion of the use of vitamin D compounds in renal failure).

Results of clinical studies in patients with secondary hyperparathyroidism undergoing maintenance hemodialysis showed that serum calcium values increased following administration of OCT in patients receiving dialysis for renal failure. However, this increase was
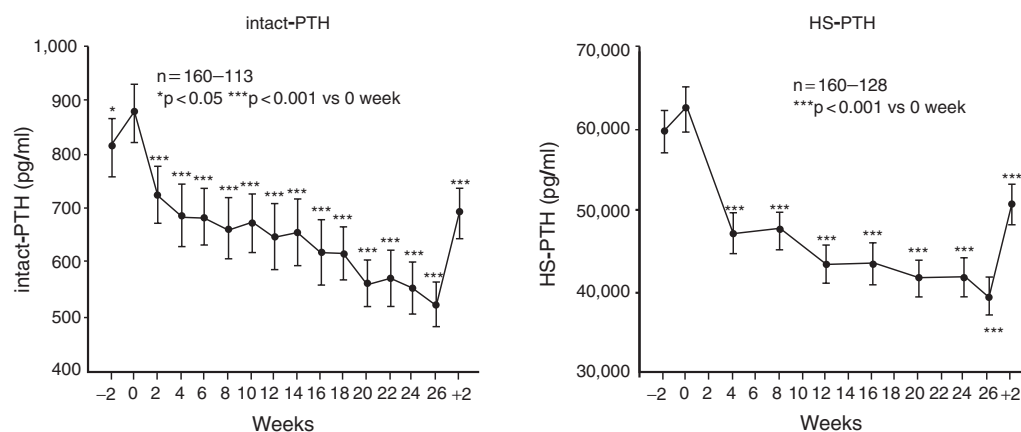
FIGURE 6   Serum intact-PTH and HS-PTH levels over time following long-term OCT administration.

remarkably modest in contrast to elevations seen with oral vitamin D analog pulse therapies currently under clinical trials. With OCT, a dose adjustment was readily accomplished and significant suppression of PTH in severe secondary hyperparathyroidism was attained. A remarkable level of PTH suppression was seen even in severe cases with intact-PTH levels as high as 1,000 to 3,000 pg/mL.

In a phase III long-term treatment study conducted with 160 chronic hemodialysis patients (97 male and 63 female; age range, 27–78 years; mean past duration of dialysis therapy, 155.3 months) having secondary hyperparathyroidism (intact-PTH level, 156-5,290 pg/mL, mean: 811.4 pg/mL; and corrected serum calcium level, 9.0–11.0 mg/dL, mean: 9.96 mg/dL), intravenous OCT injection produced a rapid decrease in serum PTH. PTH suppression was sustained over six months of treatment (Fig. 6). There were slight, yet significant, elevations in serum calcium and plasma ionized calcium values early in the course of treatment, but these elevations did not increase and were within a

controllable range by withdrawal or dosage reduction (Fig. 7). The individual dose of OCT per hemodialysis (HD) showed changes over time as shown in Figure 8. As a guide for the initial dose level of OCT, treatment was started at 10 µg/HD if intact-PTH level was >500 pg/mL or at 5 µg/HD if intact-PTH level was <500 pg/mL. The individual dose distribution showed a decline with time. While there were some cases with high PTH levels requiring dosage increases to 15 or 20 µg/HD, inconspicuous serum calcium elevations were maintained despite long-term treatment. These findings indicated the broad range of severity of secondary hyperparathyroidism.

Analysis of such data from clinical studies conducted at many institutions covering a number of patients has revealed a potent clinical effect of OCT in lowering elevated serum PTH. At the same time, the data analysis also disclosed improvement of impaired bone metabolism by the administration of OCT. Marked improvements of abnormalities indicative of high-turnover bones were noted following the treatment
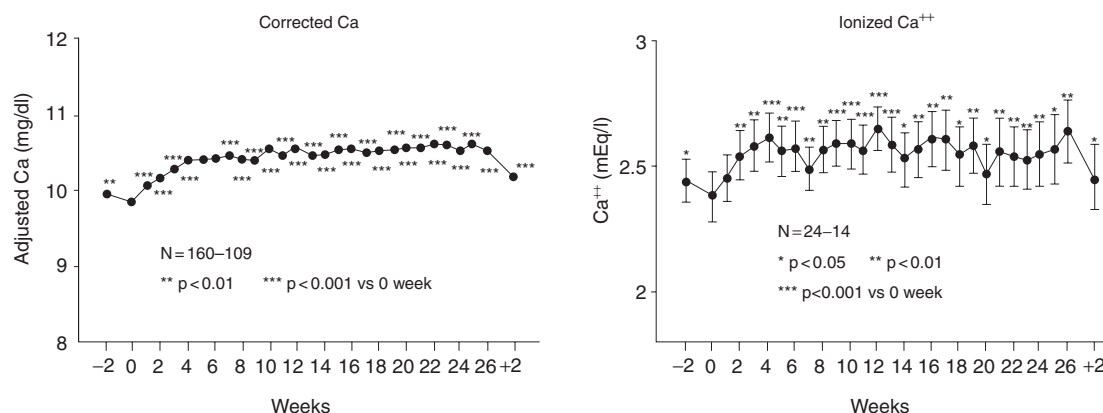

FIGURE 7   Changes in corrected serum calcium (Ca) levels and plasma ionized Ca (Ca++) concentration over time.
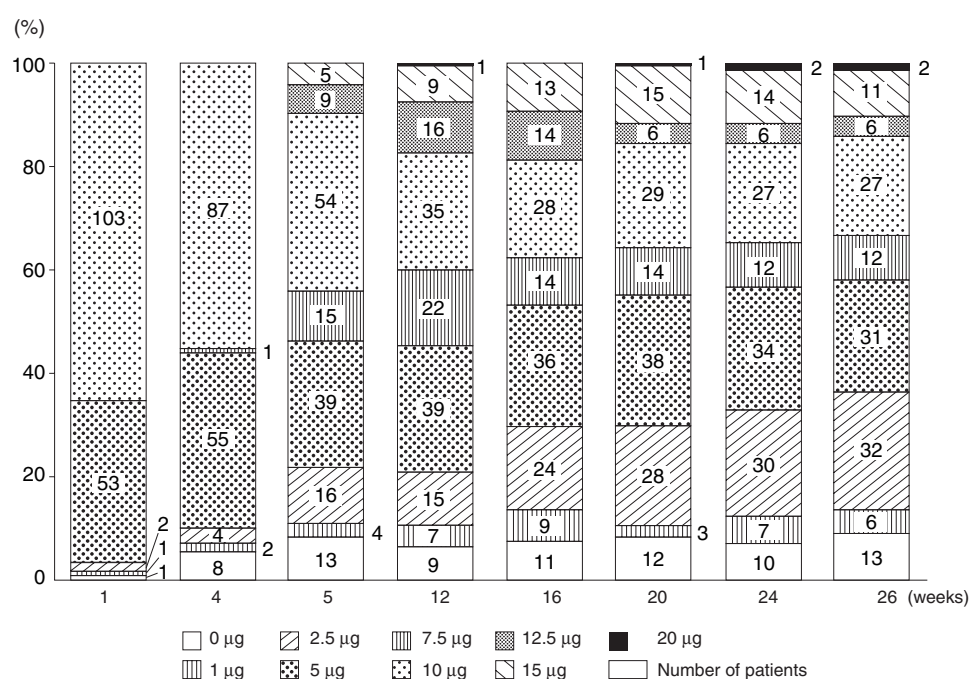
FIGURE 8 Changes in OCT dose level distribution during the course of treatment. Numbers within bars are patients that received the indicated dose of OCT.

with respect to serum/bone metabolism markers, bone resorption indices such as tartrate-resistant acid phosphatase (TRACP) and collagen type I C-terminal telopeptide (ICTP), and abnormal elevations in alkaline phosphatase(ALP)/bone ALP (BAP), procollagen type I C-terminal propeptide (PICP) and osteocalcin (BGP) [37–39] (Fig. 9).

A bone histomorphometric evaluation by Kurihara *et al.* showed reversal of high bone turnover following treatment with OCT in two patients with secondary hyperparathyroidism with "ostitis fibrosa type" skeletal changes. There was a positive increase in bone formation in one patient, hence the drug stimulated bone metabolic turnover, in the cortical bone of 2 other patients whose skeletal involvement was of the low bone turnover form, i.e., "aplastic" or "osteomalacic" type (Fig. 10). The authors stated that the enhanced bone formation observed in the low turnover bone cases was a fact of profound interest that suggested direct actions of the drug upon bone cells. Tsukamoto *et al.* reported a marked decrease in fibrotic tissue volume and improvement of high turnover bones following OCT therapy even in patients poorly responding with a serum PTH decrease [40] (Fig. 11). These findings suggest that the amelioration of osteopathy by OCT medication in severe secondary hyperparathyroidism may derive from direct actions of the drug on bone metabolism disparate from PTH-suppressive effects.

C. Psoriasis Vulgaris

Inflammatory keratoses such as psoriasis vulgaris are chronic disorders of the skin with diverse, characteristic dermatologic manifestations including erythema, thickening, cornification, and scaling (see Chapters 35 and 101). In most cases the cutaneous disorders are not completely cured and are left to symptomatic treatment. The condition of the disease often interferes with comfort and affects the patients' quality of life. The disease state is thought to be due to inflammation and abnormalities in proliferation and differentiation of skin cells. Topical corticosteroids are commonly used for treatment, and, in severe cases of the disease, therapies such as oral use of cyclosporine A and etretinate, a synthetic retinoid, may be applied. Adverse reactions to these drugs, however, are a major concern.

Topical use of active vitamin D analogs as therapeutic agents for psoriasis has been highlighted recently (see Chapter 101). Inhibition of epidermal keratinocyte proliferation, stimulation, and induction of differentiation of those cells, and immunosuppression have been implicated in the mechanisms of action for efficacy of topical active vitamin D analogs. These drugs are advantageous and show much promise as drugs of first choice in the treatment of psoriasis. They are remarkably safe and provide a longer duration time from initial cure to relapse/recrudescence than conventional therapeutic agents.
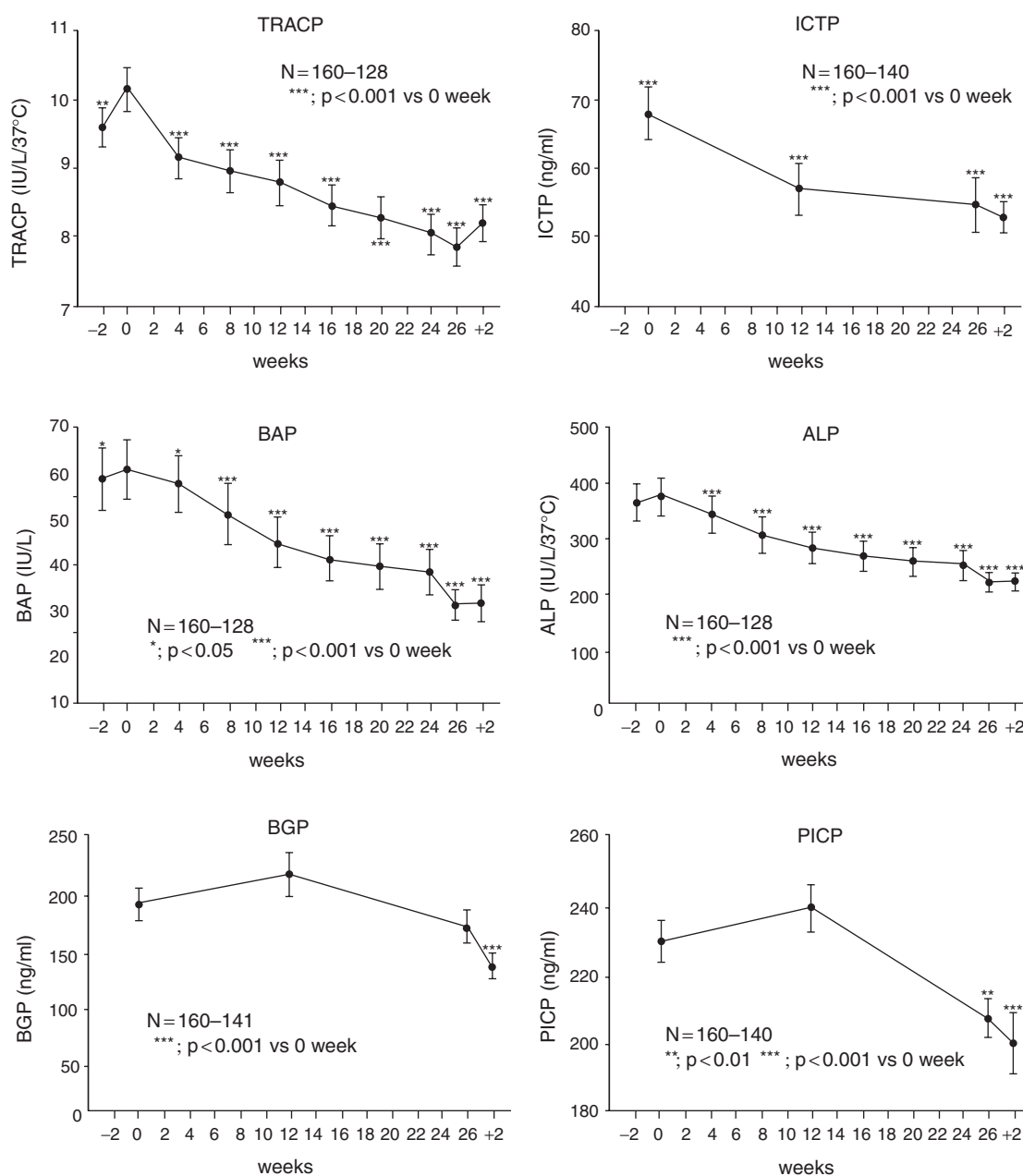
FIGURE 9 Changes in levels of bone metabolism markers following treatment with OCT. TRACP: Tartrate-resistant acid phosphatase; ICTP: Collagen type 1 C-terminal telopeptide; ALP: Alkaline phosphatase; BAP: Bone alkaline phophatase; BGP: Osteocalcin; PICP: procollagen type 1 C-terminal propeptide.

Morimoto *et al*. demonstrated *in vitro* that OCT and $1,25(OH)_2D_3$ similarly inhibited the growth of dermal fibroblasts from normal human skin in a dose-dependent manner. Growth of dermal fibroblasts from psoriatic patient skin was also inhibited by OCT in a dose-dependent fashion, but not inhibited by $1,25(OH)_2D_3$ [41].

Efficacy of OCT has been assessed primarily by *in vitro* studies because there is no established animal disease model of psoriasis. Preclinical studies including toxicology and pharmacokinetics have been carried out via percutaneous administration. Clinical trials of OCT ointment commenced in 1994. Phase I and phase II studies were conducted in Japan and the UK to evaluate safety and efficacy for topical use. To determine clinical efficacy, phase II double-blind, randomized, left vs. right, concentration-response study was
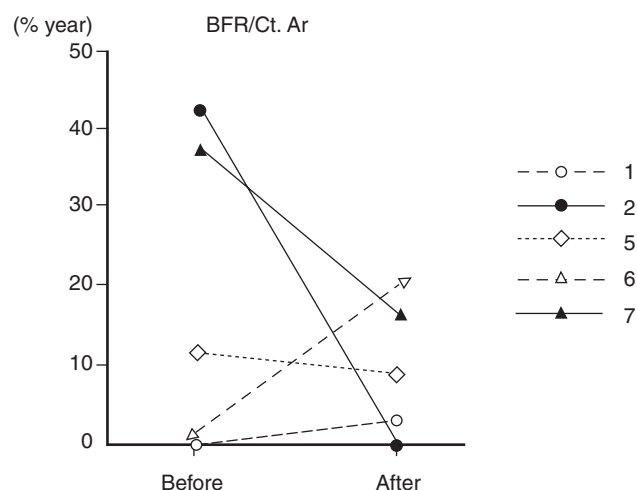
FIGURE 10   Changes in cortical bone formation rate (BFR/Ct.Ar) following administration of OCT. Cases 2 and 7: Ostitis fibrosa (high turnover); Case 5: Slight change; Case 1: Aplastic (low turnover); Case 6: Osteomalacic (low turnover)

performed with once-daily topical OCT in patients with mild to moderate chronic plaque psoriasis. Primary efficacy parameters were psoriasis severity index (PSI) based upon the sum of scores for erythema, scaling, and induration and the investigators' overall assessment of patients' response to therapy at eight weeks of treatment. In this study 144 patients participated. All concentrations of OCT ointment (6, 12.5, 25, and 50 µg/g) were significantly more effective

at reducing PSI than placebo (P<0.01), with the greatest effect noted for OCT 25 µg/g. Calcipotriol ointment 50 µg/g once daily as active comparator had a similar effect (see Chapter 84). Marked improvement or clearance of psoriasis was greatest for OCT 25 µg/g (54.7% of subjects), which compared favorably with calcipotriol (46.2%). Improvement continued throughout the study period, with no plateau at week 8. Investigators' and patients' side preference (secondary efficacy parameters) rated OCT more effective than placebo and 25 µg/g OCT better than calcipotriol (P<0.05 for investigators' assessment). Twelve patients withdrew from the study due to adverse events, of which four were judged to be due to study medication. This study indicates that once-daily OCT ointment is effective in the management of plaque psoriasis, with the greatest effect noted at a dosage of 25 µg/g. As no response plateau was seen at eight weeks, these data suggest that further benefits might be obtained if OCT ointment were applied for longer. In this study the investigators' overall assessment and general preference suggest that OCT 25 µg/g may be more effective than once-daily calcipotriol (Fig. 12).

Topical $1\alpha,24(R)$-dihydroxyvitamin D_3 (tacalcitol) preparations were previously launched and marketed in Japan as a local therapeutic agent for psoriasis while topical calcipotriol, which is already in widespread use in Europe and the United States as a drug with greater efficacy, was also marketed in Japan in 2000. Comparative studies with these topical agents have demonstrated the clinical usefulness of OCT ointment [42].

Major adverse reactions of OCT include local irritation commonly seen with topical vitamin D preparations and elevation of serum calcium following general
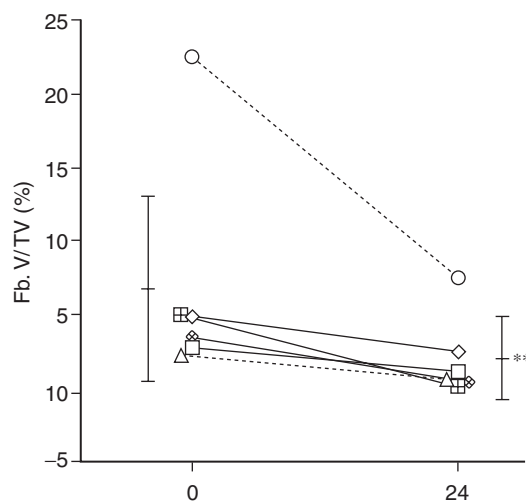
FIGURE 11   Reduction in bone marrow fibrous tissue volume following 24-week treatment with OCT. Paired t-test p<0.001; Solid line: Cases showing a ≥50% decrease intact-PTH; Broken line: Cases showing a <30% decrease intact-PTH.
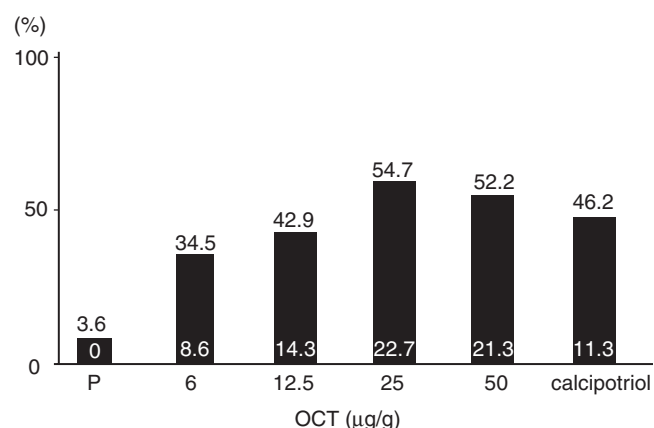
FIGURE 12   Investigators' overall assessment (primary efficacy variable) of patients' response to treatment with OCT ointment: between-patient comparison. The histogram shows results for marked improvement and clearance combined. Data for clearance alone are the numbers in white at the base of histogram. P: placebo.
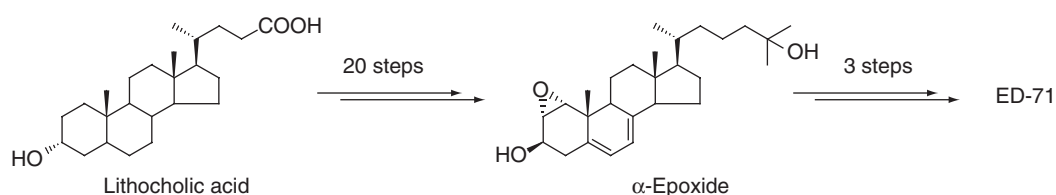
FIGURE 13   Original linear synthesis of ED-71 using lithocholic acid as a starting material.

topical application. None of the reports, however, has been serious and their occurrence is infrequent. Calcemic responses were in no case serious. Such responses are more frequent with generalized topical use of high doses early in the course of treatment with OCT. Importantly, it has been documented that safety can be secured through monitoring serum calcium levels.

III. DEVELOPMENT OF ED-71 FOR OSTEOPOROSIS

A. Convergent Synthesis of ED-71 for Versatile Method

The original synthesis of ED-71 involved a linear route using lithocholic acid as a starting material and proceeded through the α-epoxide as a key intermediate [17–18] (Fig. 13). The 23-step synthetic method seems, however, to be inconvenient for the synthesis of highly functionalized related compounds such as postulated metabolites of ED-71. We, therefore, developed a convergent strategy to ED-71 as a versatile route consisting of the A-ring and the C/D-ring fragments. The readily available C2 symmetrical epoxide was converted to the A-ring fragment in 18 steps and the C/D-ring fragment was prepared from the Inhoffen-Lythgoe diol by

known methods. Wittig reaction between the A-ring and the C/D-ring units provided ED-71 in satisfactory overall yield [43–44] (Fig. 14).

B. Preclinical Results

1. COMPARISON WITH ALFACALCIDOL

Although active vitamin D is used in certain countries for the treatment of osteoporosis, the risk of causing hypercalcemia/hypercalciuria suggests only a narrow therapeutic window. This has precluded worldwide acceptance. The results of our animal studies suggest that the therapeutic effect of active vitamin D on bone loss after estrogen deficiency can be dissociated, at least in part, from the effects of enhancing intestinal calcium absorption and suppressing parathyroid hormone (PTH) secretion [45–46]. In order to test this, we compared the effects of ED-71 with orally administered 1α-hydroxyvitamin D_3 (alfacalcidol, $1\alpha OHD_3$) on bone mineral density (BMD) and the bone remodeling process as a function of calcium metabolism and PTH levels, in an ovariectomized (OVX) model of osteoporosis. ED-71 increased bone mass at the lumbar vertebra to a greater extent than alfacalcidol (Fig. 15A). During this process calcium absorption was enhanced as indicated by urinary calcium
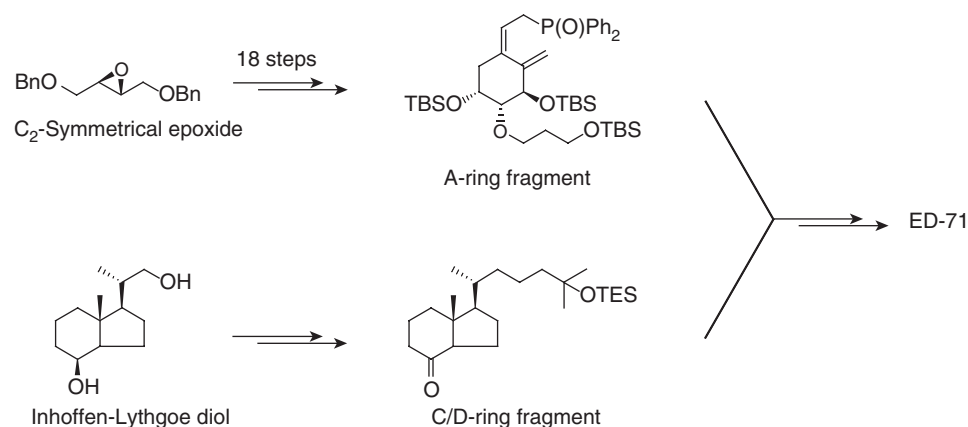

FIGURE 14   Convergent synthesis of ED-71.

excretion and serum PTH levels was decreased to the same degree as alfacalcidol. ED-71 lowered biochemical and histological parameters of bone resorption more potently than alfacalcidol (Fig. 15B), while maintaining bone formation markers (Fig. 15C). These results suggest that ED-71 exerts an anti-osteoporotic effect by inhibiting osteoclastic bone resorption while maintaining osteoblastic function, and that these anti-catabolic/anabolic effects of ED-71 take place independently of its effects on calcium absorption and PTH [47].

2. POSSIBLE MECHANISM OF ACTION OF ED-71

Drugs for the treatment of osteoporosis currently available are grossly classified into bone resorption inhibitors and bone formation stimulants. Calcitonin preparations, estrogen preparations, and bisphosphonates are defined as bone resorption inhibitors that exert therapeutic effects mainly by inhibiting osteoclastic bone resorption. PTH, on the other hand, is defined as a bone formation stimulant whose effects are mediated chiefly by its ability to stimulate osteoblasts. It is known that, with bone resorption inhibitors, bone formation is also inhibited in association with suppression of bone resorption. With PTH it is generally thought that not only bone formation but also bone resorption is stimulated. In contrast to the mechanisms of action of these drugs, it has been shown that ED-71 may have the remarkable characteristic
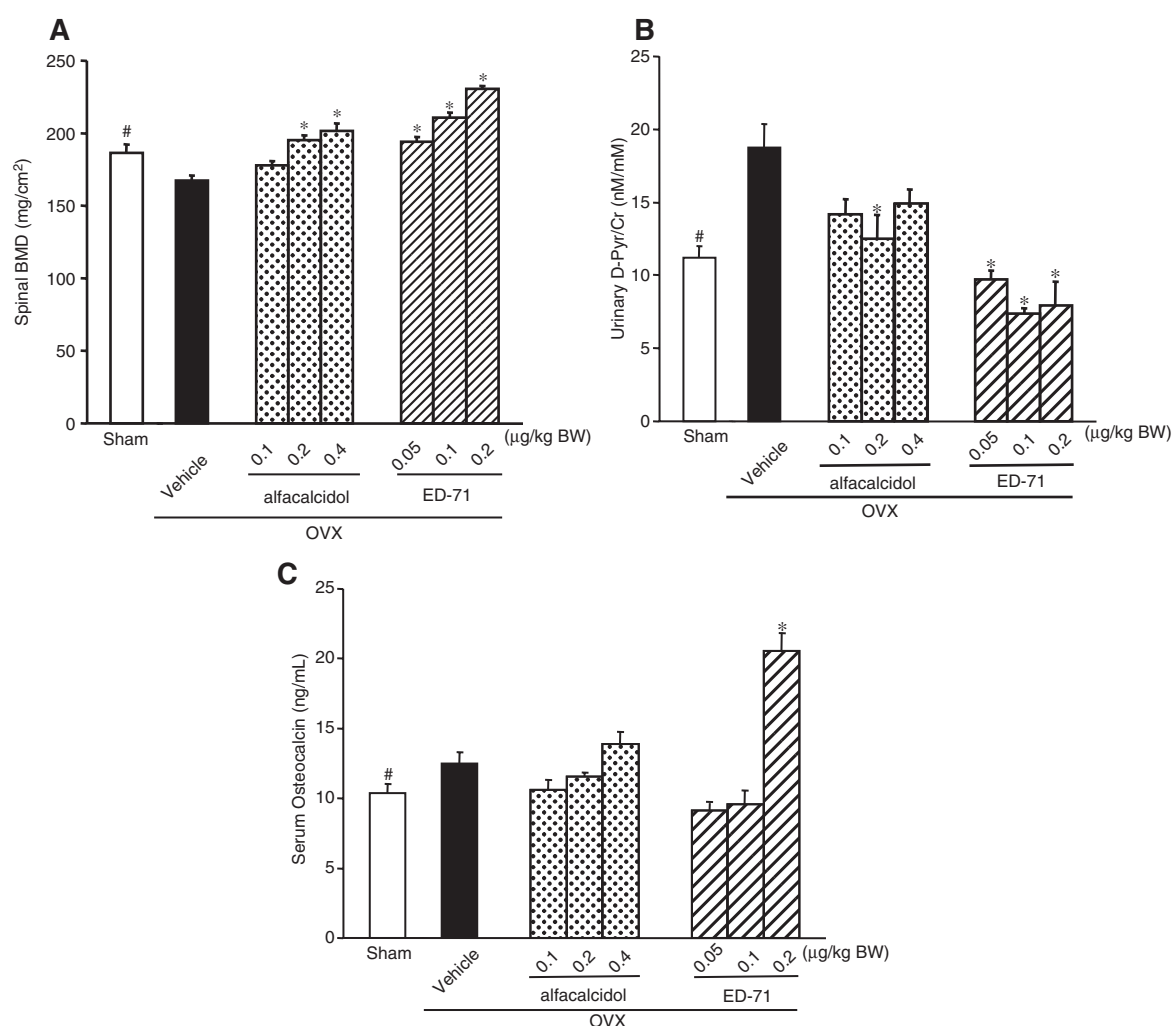
FIGURE 15 Comparison of ED-71 with alfacalcidol in ovariectomized (OVX) rats. Eight-month-old OVX Wister-Imamichi rats were treated orally with indicated doses (μg/kg BW) of ED-71 or alfacalcidol, twice or thrice per week, respectively, for 3 months. Bone mineral density (BMD) at the lumbar vertebrae (15A) was measured by dual-energy X-ray absorptiometry. Urinary deoxypyridinoline (D-Pyr) (15B) was measured using a Pyrilinks-D assay kit. Serum osteocalcin (15C) was measured using rat osteocalcin radioimmunoassay (RIA) reagents.

that it inhibits bone resorption and stimulates bone formation.

In Figure 16, changes in markers of bone metabolism observed in animals treated with ED-71 are depicted in comparison with changes following administration of 17β-estradiol. The experiment with ED-71 was performed in a therapeutic manner by initiating treatment after three months had elapsed post ovariectomy (OVX). The experiment with 17β-estradiol consisted of a prophylactic study in which the estrogenic hormone was administered s.c. for three months, starting just after an OVX, because an increase in bone mass could not be anticipated in this system. Urinary deoxypyridinoline excretion (Dpyr/Cr), a bone resorption parameter, and osteocalcin (BGP),

a bone formation parameter, both decreased dose-dependently to below the sham-operated group level in response to administration of 17β-estradiol [46]. As a result, the decrease in BMD due to ovary anatomy was inhibited, such that the BMD was maintained at the sham-operated group level but showed no further increase.

In the animals treated with ED-71, enhanced urinary Dpyr/Cr was lowered to below the sham-operated group level as in the 17β-estradiol treated group while BGP was increased in a dose-dependent fashion. Animals given PTH displayed a dose-dependent increase in BGP but no trend in increasing urinary Dpyr/Cr was observed (data not shown in this figure).
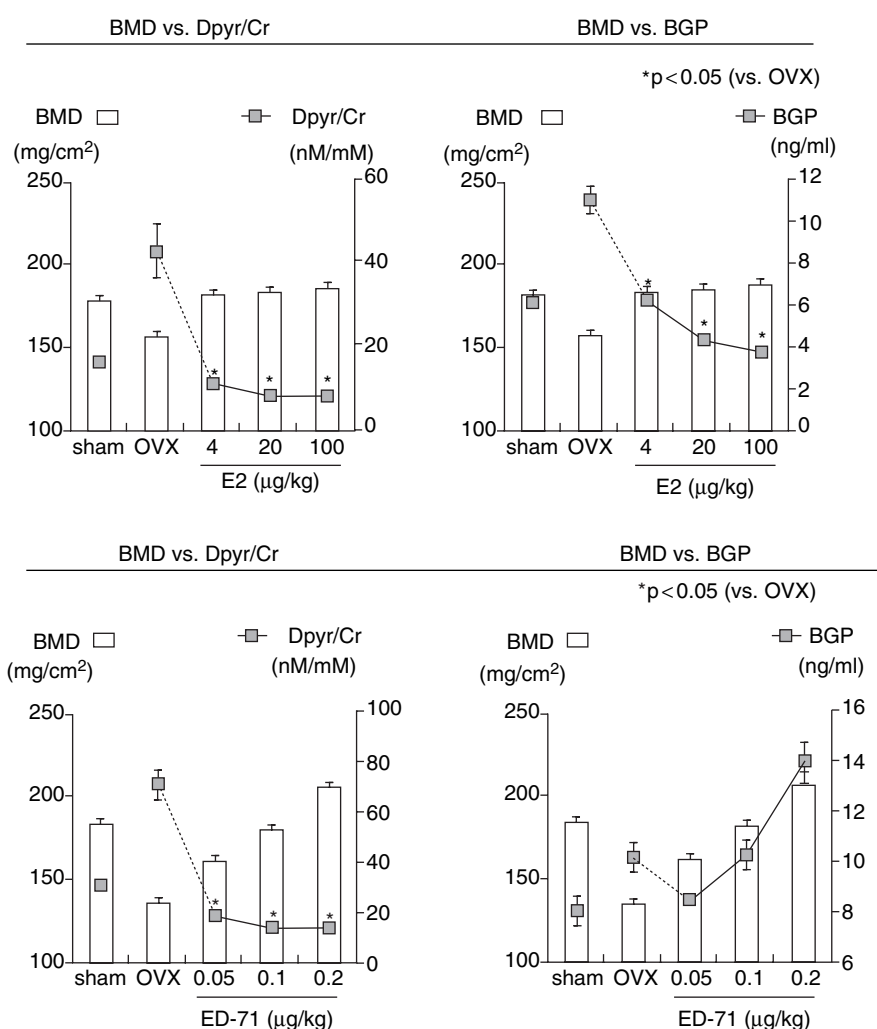

FIGURE 16   Changes in bone metabolism parameters in 17β-estradiol (E$_2$) treated groups and ED-71 treated groups. Data are presented as the mean ± SE, *p < 0.05 (vs. Ovariectomy (OVX), Dunnett's multiple comparison). BMD: Bone mineral density; Dpyr/Cr: Urinary deoxypyridinoline excretion; BGP: Osteocalcin.

Results of bone histomorphometric analysis made on hard tissue preparations of the third lumbar vertebra from animals are shown in Fig. 17. In the ED-71 treated group, there was a decrease in osteoclast surface (Oc.S/BS) to below the sham-operated group level while bone formation rate (BFR/BS) was increased to above the OVX group level. There was also an increase in mineral apposition rate (MAR) in these animals; thus bone formation was definitely stimulated.

Regarding the bone formation stimulant effect of ED-71, Tsurukami *et al*. reported that ED-71 produced increases in bone mass and bone formation rate without reducing the trabecular unit activation frequency. This was inferred from bone histomorphometric indices for the fifth lumbar vertebrae of OVX rats having been given ED-71 for 12 weeks [48]. When normal rats of which skeletal growth had ceased due to aging were dosed with ED-71 for 12 weeks, subsequent bone histomorphometric examination revealed a significant increase in lumbar vertebral bone density and dose-dependent increases in total osteoid surface (OS/BS), MAR, and BFR/BS. These results provide supportive evidence for the stimulation of bone formation by ED-71 *in vivo*.

The above data demonstrate that ED-71 may have a uniquely characteristic mechanism of action whereby it inhibits bone resorption and also stimulates bone formation. This mode of action is unlike that displayed by currently available bone resorption inhibitors, which are known to inhibit bone resorption and also to suppress bone formation. Furthermore, bone formation stimulants such as PTH accelerate not only bone formation but bone resorption as well (Fig. 18).

At present, drugs which inhibit bone resorption constitute the primary therapeutic modality in the medical treatment of osteoporosis. Generally, patients with osteoporosis often have a decrease in bone mass, subjective symptoms, and intercurrent fractures of vertebral bodies. Through the use of drugs which inhibit bone resorption, halting the progression of the disease may be achievable but the diminished bone mass cannot be restored. Active vitamin D analogs also appear to have a superior efficacy in reducing incidence of fractures in osteoporotic patients compared to their efficacy in maintaining/increasing bone mass. It would be probable that the incidence of fractures may be further reduced if an active vitamin D analog possessing potent bone mass-increasing activity were to become available. Since ED-71 appears capable of restoring decreased bone mass via its bone formation inducing activity, it serves as a potentially promising, ideal therapeutic agent for osteoporosis.

C. Clinical Results

1. PHASE I

Phase I clinical trial of ED-71 was conducted to estimate safe dosage levels and assess the pharmacokinetics of ED-71 in healthy adult male volunteers. There were no findings of clinical concern, including a calcemic effect, resulting from a single-dose administration study at dose levels of 0.01 to 1.0 µg. In a repeated-dose administration study using dosages of 0.1, 0.25, 0.5, and 1.0 µg once daily for 15 days, the following observations were noted:

1. There was a dose-related increase in serum concentration of ED-71, and the concentration reached a steady state by day 13 of administration, showing linearity within a dose range of 0.1 to 1.0 µg.
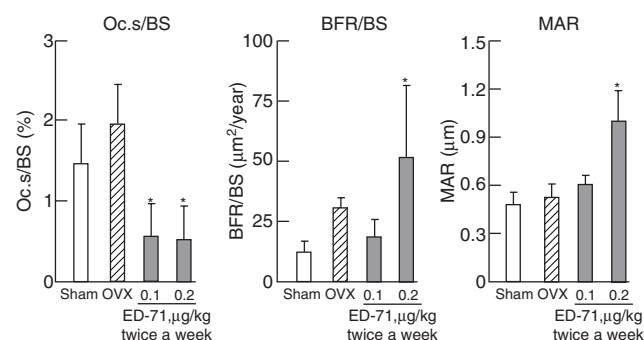
FIGURE 17 Bone histomorphometric data for lumbar vertebrae of ovariectomized (OVX) rats following treatment with ED-71. 8-month-old female Wistar rats were ovariectomized and, upon confirming a sufficient bone mass decrease after 4 months post-operation, they were administered ED-71 twice weekly for 3 months. Data are presented as the mean ± SE. Oc.s/BS: Osteoclast surface; BFR/BS:Bone formation rate; MAR: Mineral apposition rate.
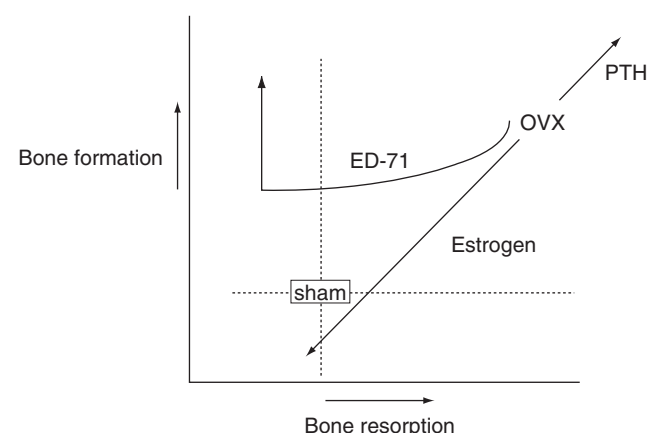
FIGURE 18 Possible mechanism of action of ED-71.

2. The earliest pharmacological reaction that appeared was an increase in urinary calcium excretion per day. This increase was dose-dependent with no evidence of a change in serum calcium concentrations.
3. Bone resorption markers decreased in a dose-dependent manner while bone formation markers showed no significant change.
4. PTH suppression was not observed even at dose levels of the drug that caused a significant decrease in plasma $1\alpha,25$-dihydroxyvitamin D concentration.

The above results demonstrate that ED-71 effectively inhibits bone resorption without suppressing bone formation at nonhypercalciuric/nonhypercalcemic dosage levels.

2. EARLY PHASE II

In early phase II clinical trials, a randomized controlled study with ED-71 in 109 osteoporotic patients (102 females and 7 males), 49 to 81 years of age (mean 65.0 years) was conducted. The patients were randomly assigned to either 0.25, 0.5, 0.75, or 1.0 µg/day of ED-71 administered orally. They were treated for six

months, and BMD and bone markers were evaluated. ED-71 treatment increased BMD in the lumbar spine (L_{2-4}) in a dose-dependent manner $(0.34\pm0.73, 0.50\pm0.91, 3.00\pm0.65$ and $2.66\pm0.71\%$ in the 0.25, 0.5, 0.75 and 1.0 µg groups, respectively, mean $\pm$ SE), and the effect of ED-71 reached the peak at 0.75 µg (Fig. 19). The percentages of patients that showed an increase in the L_{2-4} BMD over 3% after six months also increased dose-dependently (21.7, 26.1, 54.2, and 45.5% in the 0.25, 0.5, 0.75, and 1.0 µg groups, respectively). ED-71 exhibited dose-dependent suppression of urinary deoxypyridinoline and Crosslaps excretion as well as serum bone-type alkaline phosphatase, whereas serum osteocalcin was not suppressed, suggesting maintenance of bone formation with suppression of bone resorption. Serum level of $1\alpha,25$-dihydroxyvitamin D was reduced dose-dependently and 24,25-dihydroxyvitamin D levels were increased by the treatment of ED-71. These findings reveal that ED-71 treatment inhibits the activation of native vitamin D and accelerates the metabolism of activated vitamin D. Intact PTH levels were not remarkably suppressed by ED-71 compared to the treatment levels. At present we do not know why
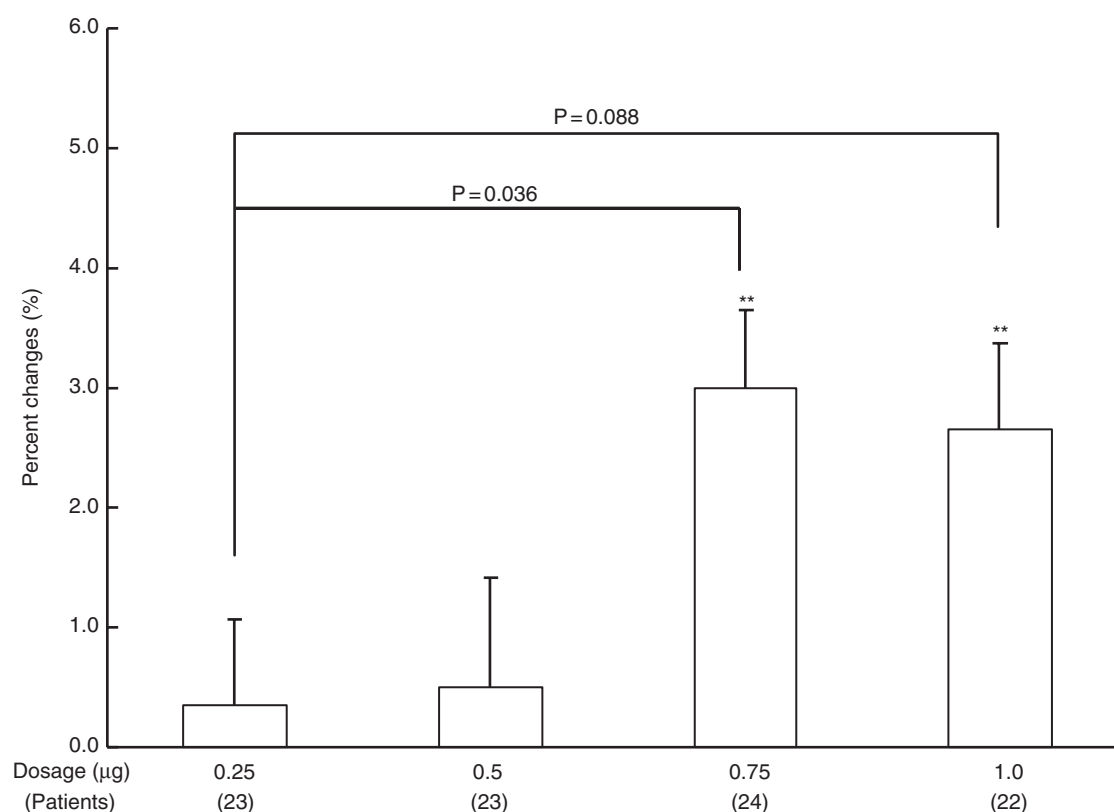

FIGURE 19   Percentage change in lumbar spine (L_{2-4}) bone mineral density (BMD) after 24 weeks treatment of ED-71. **p<0.01: Paired t-test (Dunnett's test) between groups.

ED-71 is unable to suppress PTH levels, although this might be due to a unique property of ED-71 compared to active vitamin D_3 compounds such as $1,25(OH)_2D_3$ and alfacalcidol. ED-71 was well-tolerated without causing hypercalcemia, and no patient exhibited sustained postprandial hypercalciuria over 0.4 mg/dL GF. These results support the need for further long-term clinical studies to examine the effects of ED-71 on bone fracture in osteoporotic patients [49–50].

3. Late Phase II

Based upon the results of the early phase II trials with ED-71, we are now conducting late phase II further clinical studies as with the following protocol:

- Patients: primary osteoporosis 200 patients
- Design: randomized double-blinded study
- Doses: 0, 0.5, 0.75, and 1.0 µg/day
- Treatment; once a day for successive 48-week treatment 400 or 200 IU vitamin D_3 is added depending on initial 25-hydroxyvitamin D levels
- End points at 48 weeks: lumbar spine (L_{2-4}) BMD and bone markers

The above-mentioned late phase II study will be completed until summer of 2003 and the subsequent phase III study for fracture prevention is scheduled.

Acknowledgments

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2-Carbon-Modified Analogs of 19-Nor-1α,25-Dihydroxyvitamin D₃

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I. INTRODUCTION

With the discovery of the many functions of vitamin D beyond calcium homeostasis and phosphate metabolism has come the search for the holy grail of analogs. This search is largely aimed at analogs of 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) that produce no hypercalcemia or hyperphosphatemia while still retaining activities such as suppression of parathyroid gland proliferation and preproparathyroid hormone expression, the regulation of keratinocyte proliferation and gene expression, inducing of apoptosis in cancer, and suppression of proliferation by malignant cells. This has been the driving force in the chemical synthesis of a large variety of analogs. At least partial success has been achieved for a variety of different reasons. Side-chain modifications have been the particular target of synthesis. In our laboratory, elongating the side chain of the 24-carbon proved to reduce *in vivo* activity of stimulating intestinal calcium transport and bone calcium mobilization while at the same time retaining or increasing activity in the cellular differentiation assay using HL-60 cells in culture [1–3]. Similar results were obtained with the MC-903 compound of Leo Pharmaceuticals [4] (see Chapter 84), 22-oxa-1α,25-dihydroxyvitamin D₃ of

Chugai [5] (see Chapter 86) and the 16-ene derivatives of Hoffmann-La Roche [6] (see Chapter 85). Unfortunately, these deductions were based on *in vitro* assays in the case of cellular differentiation and *in vivo* assays in the case of calcium mobilization and serum calcium elevation. Nevertheless, it resulted in compounds that appeared to be much less calcemic *in vivo,* but that retained activity in cellular differentiation and presumably in other cellular activities. Seizing on this opportunity, Leo Pharmaceuticals successfully developed MC-903 for the topical treatment of psoriasis, providing a highly successful drug, Dovonex™ [7] (see Chapter 101). Of great interest is that these compounds are largely noncalcemic because they are rapidly metabolized and excreted, thereby eliminating their activity *in vivo* [8,9]. Thus, these derivatives, although active when applied topically, have very low activity when provided systemically. The 22-oxa derivative appears to retain sufficient lifetime in the plasma to suppress parathyroid activity and it has found use as an analog for the treatment of bone disease secondary to kidney failure [10]. Thus, the primary discrimination in the case of these analogs is rapid metabolism and elimination once they enter the circulation. This does not result in a truly noncalcemic analog, but one that is

discriminated on the basis of metabolism. However, other side-chain alterations have proven to be very useful as described below in the case of some specific analogs. One of the important lessons to be learned from the early work on analogs is that the compounds must be tested *in vivo* for all activities before firm deductions can be made regarding selectivity of activity. Thus, the quest still remains for a noncalcemic form of $1,25(OH)_2D_3$ that nevertheless can act *in vivo* to suppress cancer, to suppress parathyroid secretion, and to suppress keratinocyte proliferation. They may also be useful for the treatment of autoimmune diseases as well.

The following chapter will be devoted to the development of some specific 2-carbon-modified analogs of 19-nor-$1,25(OH)_2D_3$ that show promise for selectivity for bone and which may provide new analogs that are devoid of calcium activity while still functioning *in vivo* in suppression of parathyroid hormone or acting on the keratinocyte.

II. 19-NOR- AND 10,19-SATURATED DERIVATIVES OF $1,25(OH)_2D_3$

An important ingredient in the development of the 2-carbon-modified analogs that will be described in this chapter was the finding of the lack of importance of the 10,19-methylene carbon for the function of $1,25(OH)_2D_3$. Before the advent of a crystal structure or computational models of the vitamin D receptor (VDR), one interest in our laboratory was to determine whether the cis-triene structure of $1,25(OH)_2D_3$ is essential for function. As a result, 19-nor-$1\alpha,25$-dihydroxyvitamin D_3 was chemically synthesized [11]. The molecule and related derivative were then tested for biological activity *in vivo* as well as *in vitro*. These compounds retained the ability to bind to the VDR, did not alter the ability to induce *in vitro* transcription using reporter genes and the 24-hydroxylase promoter, but appeared to be 1/25th as active as $1,25(OH)_2D_3$ in raising serum calcium and serum phosphorus. Of considerable interest is that when the 19-nor-$1,25(OH)_2D_3$ derivative was further derivatized by elongating the side chain by the addition of methyl groups on the 26- and 27-carbons or by the provision of 2-methylene groups providing a 5-membered ring surrounding the 25-hydroxyl, these compounds repossessed their calcemic activity [12]. Thus, it is very clear that the 10,19-methylene group is not required for the functions of vitamin D. Furthermore, if the side chain is either the cholesterol or ergosterol side chain, it has reduced calcemic activity. This resulted in the development of

the important new drug, paracalcitol or Zemplar®, for the treatment of renal osteodystrophy [13]. The advantages of this compound over calcitriol itself are that it is much less calcemic, but only slightly less active in suppression of parathyroid tissue and parathyroid secretion. Thus, the use of Zemplar® has provided the nephrologists with an important treatment of secondary hyperparathyroidism of renal osteodystrophy with a markedly increased window of safety (see Chapter 76). The value of this compound has recently been confirmed by an impressive 50,000-patient evaluation of its success versus that of calcitriol [14]. Thus, the 19-nor derivatives represent a true *in vivo*-active compound with reduced calcemic activity.

In pursuit of further understanding of the lack of importance of this derivatization to the action of vitamin D, the 10,19-dihydro-$1\alpha,25$-dihydroxyvitamin D_3 compound was prepared. It was found to be much less active in all respects [15]. The methyl group in that position alters significantly the ability of the A-ring to assume an appropriate conformation and thus did not provide the necessary information in regard to the importance of the 10,19-methylene group. Finally, the 6,7-aza-$1\alpha,25$-dihydroxyvitamin D_3 derivative was prepared and was also devoid of biological activity despite the fact that it retained the π electrons that would be provided by the diene structure of the 19-nor-$1,25(OH)_2D_3$ [16]. It appears that the diene structure is absolutely essential for the function of the vitamin D hormone. Furthermore, this has been confirmed by the exact alignment in crystal structure of the 5–6 and 7–8 double bonds with the tryptophan π electrons and also explains the absorption shift of the tryptophan residue when the ligand is bound.

III. EPIMERIZATION OF THE 20-CARBON

The 20-carbon epimerization of $1,25(OH)_2D_3$ has been studied largely by Leo Pharmaceuticals [17]. Furthermore, Wicha and colleagues (unpublished) have studied the epimerization of the 20-carbon of vitamin D. The epimerization of the 20-carbon appears to play little or no role in binding to the receptor, but significantly increases biological activity both *in vivo* and *in vitro* [18]. This derivatization does not appear to provide selectivity in terms of the known actions of vitamin D but merely will increase under some circumstances biological activity by severalfold. This epimerization has been incorporated into the new selective 2-carbon-modified analogs described here.

IV. EARLY 2-CARBON ANALOGS OF 19-NOR-1,25(OH)$_2$D$_3$ AND 25-OH-D$_3$

An important breakthrough was that provided by Nishii *et al.* in which it was shown that the 2β-substitution of 2β(hydroxy)-3′-propylalcohol (ED-71) was well tolerated in binding to the VDR and provided activity that seemed to favor bone calcium mobilization [19]. Most important, it illustrated that large substitutions on the 2-carbon could be made without interfering with binding to the receptor and subsequent biological activity. This led to an investigation of 19-nor vitamin D compounds in our laboratory which were substituted with the 2α- and 2β-hydroxyls. In this study, the 2β-hydroxylated compound was slightly better than the 2α-hydroxylated compound, but nevertheless they both provided significant *in vivo* biological activity [20].

This was followed by an investigation into the substitution of larger groups. In particular, the presence of the 2-benzyloxy ether in the 2α-position not only was well tolerated but provided high intestinal calcium transport activity with relatively low bone calcium mobilizing activity [20].

The Chugai compound ED-71 is currently being developed for the treatment of osteoporosis (see Chapter 86). These compounds all led to our investigation of substitutions on the 2-carbon position of 19-nor-1,25(OH)$_2$D$_3$ compounds.

V. 2-METHYLENE AND 2α-METHYL AND 2β-METHYL DERIVATIVES OF 1,25(OH)$_2$D$_3$

In this laboratory, having learned that the diene system is required and having learned that side-chain modifications except the 20-epimerization diminishes *in vivo* activity, our focus on modification of 1,25(OH)$_2$D$_3$ turned to the A-ring. By removing the 10,19-methylene group, we learned that we could reduce *in vivo* calcium activity provided the side chain was not appreciably altered [11,21]. Finally, we began to consider possible derivatizations that could alter the activity of 1α- and 3β-hydroxyl functionalities, which appeared to play an important role not only in binding to the VDR but in biological activity. Thus, the series of compounds, the most interesting of which are shown in Fig. 1, were chemically synthesized and studied extensively for biological activity. Of particular importance was the 2α-methyl derivative that appeared to lock the 1-hydroxyl in the axial configuration. This appeared to be interesting because there had been proposals that the binding form to the receptor required this hydroxyl to be in the equatorial form [22].
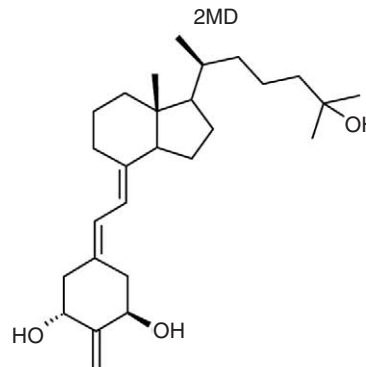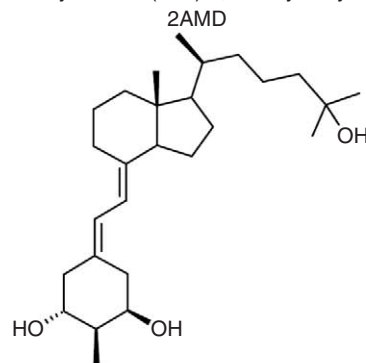
FIGURE 1 2-Carbon-modified analogs of 1,25(OH)$_2$D$_3$. The 2-methylene-19-nor-(20S)-1α,25-dihydroxyvitamin D$_3$ (2MD) and the 2α-methyl-19-nor-(20S)-1,25-dihydroxyvitamin D$_3$ (2AMD) represent two potent bone selective analogs of 19-nor-1α,25-dihydroxyvitamin D$_3$. The 2β-methyl compound (2BMD) has much weaker activity both *in vitro* and *in vivo*.

We, therefore, synthesized the following series of compounds: 2-methylene-19-nor-(20S) and (20R)-1α,25-dihydroxyvitamin D$_3$; 2α-methyl-19-nor-(20S) and (20R)-1α,25-dihydroxyvitamin D$_3$; and 2β,19-nor-(20S) and (20R)-1α,25-dihydroxyvitamin D$_3$ and studied their

biological activities. It became immediately apparent that the 2β-methyl derivative failed to produce an analog of interest since it failed to bind to the VDR and consequently had very low biological activity. However, the 2-methylene and 2α-methyl derivatives both proved to be extremely potent analogs of $1,25(OH)_2D_3$. These derivatives bind to the VDR as well as $1,25(OH)_2D_3$ [23]. They are also able to induce differentiation of HL-60 cells with the 20S derivatives being much more active than the 20R derivatives, both being at least equal or more active than $1,25(OH)_2D_3$. These compounds also proved to be quite active in stimulating intestinal calcium transport; however, their activity in that regard ultimately appeared to approximate that of $1,25(OH)_2D_3$ [24]. However, the major departure from the native hormone is with the 20S derivatives especially but also to some degree with the 20R derivatives. These compounds are much more active in mobilizing calcium from bone. One of these has been tested more fully in this system as shown in Fig. 2. As a result of a log dose study, it is clear that 2-methylene-19-nor-(20S)-$1,25(OH)_2D_3$ is equal to the activity of $1,25(OH)_2D_3$ in intestinal calcium transport, but is approximately 30–100 times more active than $1,25(OH)_2D_3$ in mobilization of calcium from bone. In this latter assay, animals are made both vitamin D–deficient and deprived of dietary calcium. Thus, in the absence of calcium, they are unable to synthesize new bone. The administration of vitamin D compounds under these circumstances causes calcium to come from bone to provide for serum calcium concentrations. This, therefore, represents an *in vivo* measurement of osteoclastic-mediated bone resorption. Thus, especially with the 2-methylene-19-nor-(20S)-$1,25(OH)_2D_3$, we see true selectivity for activity on the skeleton *in vivo*. This study was then a forerunner of a series of 2-carbon analogs being developed in our laboratories.

FIGURE 2   2MD is approximately 30 times more potent than $1,25(OH)_2D_3$ (calcitriol) in the mobilization of calcium from (**A**) bone and is equally potent in stimulating (**B**) intestinal calcium transport. In these experiments, vitamin D–deficient rats were provided the indicated doses i.p. daily for four days and on the 5th day, serum calcium determinations were made and intestinal calcium transport determinations were performed by the everted sac technique. The rise in serum calcium must arise from the skeleton because of the lack of calcium in the diet. This activity is via the stimulation of osteoblasts to produce RANKL that activates osteoclastogenesis.

VI. 2-METHYLENE-19-NOR-(20S)-$1,25(OH)_2D_3$ (2MD) AND 2α-METHYL-19-NOR-(20S)-$1,25(OH)_2D_3$: ANALOGS THAT POSSESS ANABOLIC ACTIVITY ON THE SYNTHESIS OF BONE AND APPEAR TO BE BONE SELECTIVE ANALOGS OF $1,25(OH)_2D_3$

The above studies indicated bone selectivity for 2MD and 2αMD. Because of the cost of pursuing both compounds, efforts were placed entirely on 2MD, but there is reason to believe that the 2α-methyl derivative would behave similarly. To pursue the bone selectivity further, Shevde *et al*. [24] studied osteoclastogenesis *in vitro* utilizing bone marrow culture. The results

illustrate that 2MD has an activity two orders of magnitude greater than $1,25(OH)_2D_3$ in osteoclastogenesis, presumably by inducing the synthesis of RANKL. This is truly a remarkable increase in biopotency for a bone system. This led to the natural question of whether all activities of the osteoblast might be stimulated by 2MD. Thus, Shevde *et al*. [24] incubated 2MD with human osteoblast cultures taken from discarded bone sutures from pediatric cases. In this system, the osteoblasts were incubated with various concentrations of 2MD for 7 days and then incubated with β-glycerol phosphate and ascorbic acid to cause bone nodule formation. The results shown in Fig. 3 show clearly that 2MD is not simply two orders of magnitude more active because it shows this activity whereas $1,25(OH)_2D_3$ under the
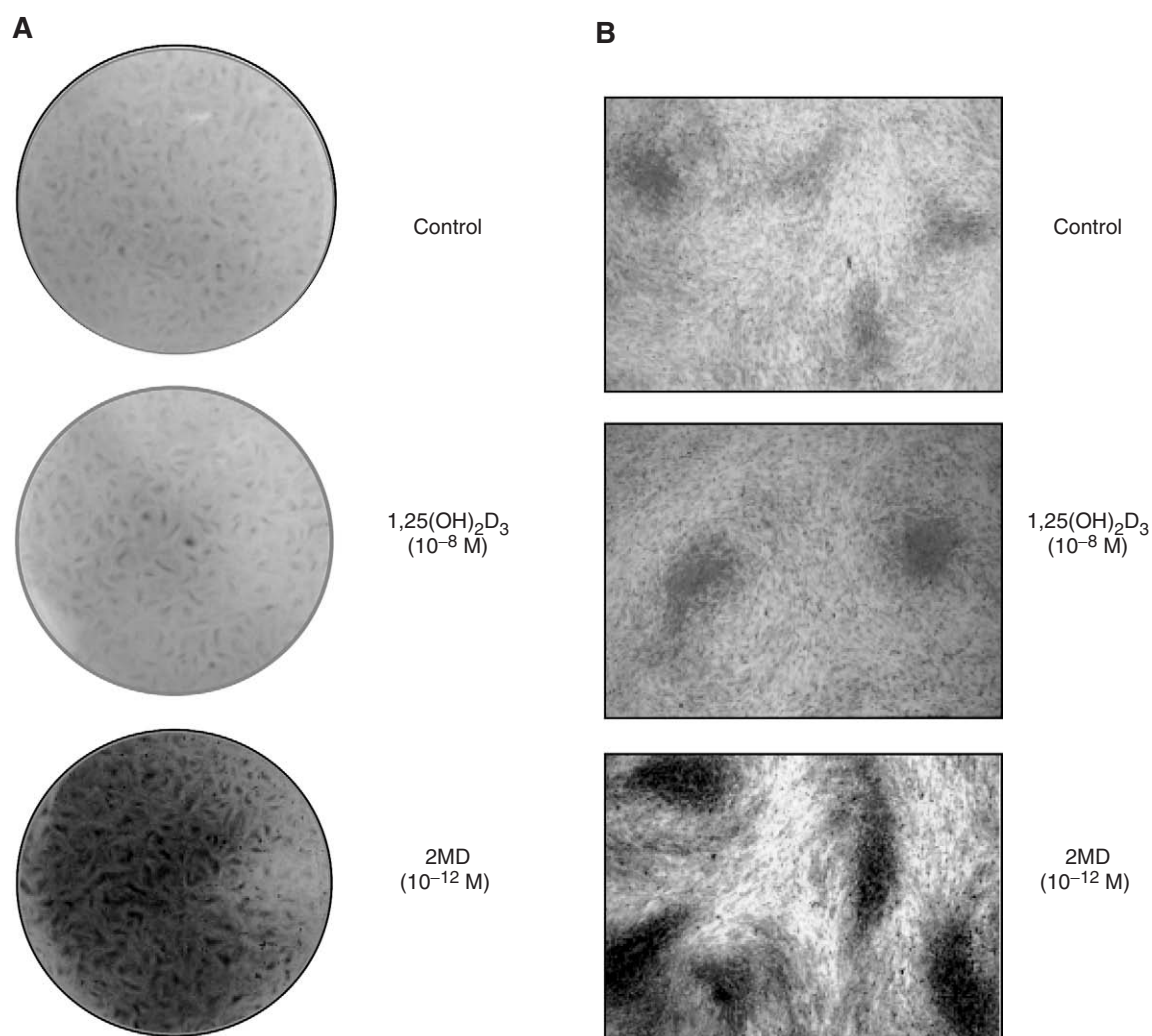
FIGURE 3   2MD induces potent mineralization in osteoblasts *in vitro*. Primary human osteoblasts were isolated and cultured at a density of 3×10^5 cells/ml. Confluent cultures were treated with $1,25(OH)_2D_3$ or 2MD at the concentrations indicated in the figure on days 0, 3, and 6 followed by treatment with ascorbic acid and β-glycerophosphate on days 9 and 12. Cells were stained on day 14 using the Von Kossa staining technique to detect the presence of calcified matrix/bone. The dark brown to black stain is indicative of calcified bone nodules. Von Kossa-stained cultures are indicated on the left (**A**) and microscopic images (10×) are shown on the right (**B**).

same circumstances has little or no activity. Thus, in an *in vitro* system, this analog demonstrates marked bone anabolic activity.

Because of the bone selectivity, experiments were initiated early on to determine if 2MD could increase bone mass in the ovariectomized, aged female rat retired breeder. By now, more than five *in vivo* experiments have been conducted, all with similar results [24]. A representative example is shown in Fig. 4. After 23 weeks of treatment at 5–7 ng/kg/body weight, 2MD causes a 10% increase in total body bone mineral density. In data not shown, this increase is in both trabecular and cortical bone, each showing in the case of the femur, a 25% increase in trabecular and cortical bone. Bone strength measurements show that this skeleton has the expected strength as related to bone mineral density. Finally, bone histomorphometry has demonstrated that this increase in bone mass is the result of increased bone synthesis.

As a result, 2MD is now under development as a pharmaceutical for the treatment of postmenopausal osteoporosis. We believe this compound can be used wherever bone synthesis is required, i.e., osteoporosis or fracture healing or prosthesis with the skeleton.
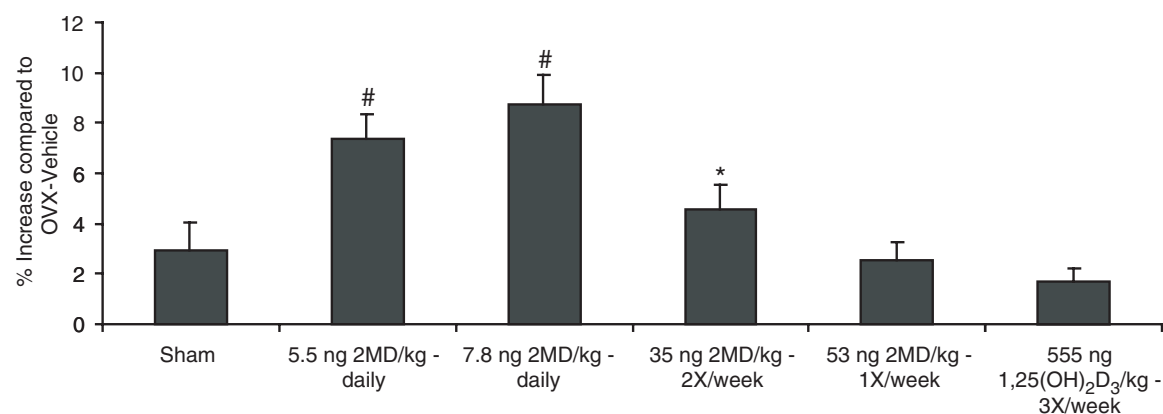
Values shown are the average of 8–12 animals/group. Error bars represent standard error. Statistically significant ($p < 0.05$) values are denoted by pound signs (different from both sham and OVX-vehicle control animals) or asterisks (different from OVX-vehicle control animals).

FIGURE 4　One-year-old female retired breeder rats obtained from Sprague Dawley were either sham-operated or ovariectomized. After 5 wk, the animals were given the indicated oral doses of 2MD or $1,25(OH)_2D_3$ for a period of 23 weeks. The percent increase of total body bone mineral density in the animal as compared to the ovariectomized controls is plotted. Clearly, 5–6 ng of 2MD/kg/day produced an astounding 8–9% increase in total body bone mineral density. Twice weekly dosing was less effective and even less effective was once-a-week dosing. Notice that a very much larger dose of $1,25(OH)_2D_3$ given three times a week only increased the bone mass to about the level of the sham-operated control and thus gave a very modest percent increase over the ovariectomized control. Bone histomorphometry and other measurements have indicated a 25% increase in trabecular bone, and this increase was not the result of an inhibition of resorption but rather resulted from a significant increase in new bone formation.

VII. 2-METHYLENE-19-NOR-(20S)-1,25(OH)$_2$D$_3$: MOLECULAR MECHANISMS OF TISSUE SELECTIVITY AND ENHANCED POTENCY

The 2 carbon-modified analogs of 19-nor-1,25–$(OH)_2D_3$ and the many additional analogs discussed in various chapters in this book show tremendous potential for the effective treatment of many diseases. Indeed, 2MD is currently in development as a treatment for osteoporosis, and other analogs are also being developed for a variety of other indications. Despite this, the mechanisms that underlie the unique actions of most of these compounds relative to altered potency and/or efficacy, or in some cases tissue selectivity *in vivo* remain largely unknown. The fundamental unanswered question is whether these analog-specific patterns of biological activity observed *in vivo* are due to differences in analog activity within target cells (Chapter 83), or are due to unique *in vivo* differences in compound metabolism, pharmacokinetic behavior, and/or tissue localization (Chapters 81 and 82). Efforts are currently underway in various laboratories to answer these questions for each individual analog.

With respect to 2MD, it and likely other 2 carbon-modified $1,25(OH)_2D_3$ analogs are exceptionally active in the skeleton *in vivo*, and appear to favor activities that lead to striking net bone formation rather than the modest or, in some cases, neutral effects that $1,25(OH)_2D_3$ exhibits on bone. As indicated earlier in this chapter, although the overall mechanism through which 2MD functions in this anabolic fashion is unknown, perhaps the most informative discovery is the finding by Shevde *et al*. [24] that 2MD exerts a unique and highly potent mineralizing effect on differentiating osteoblasts *in vitro* at concentrations well below those that efficiently stimulate the formation and activation of osteoclasts (Fig. 3 and ref. 24). Since the osteoblast plays a central role in both bone forming and bone resorbing activities and is the principal target of vitamin D action in bone [25], these data suggest that 2MD may activate a gene or genes in osteoblasts that are unique relative to $1,25(OH)_2D_3$. Studies are in progress to identify these gene targets and to assess their role(s) in bone formation both *in vitro* and *in vivo*.

Perhaps the most obvious feature of 2MD, and indeed a characteristic typical of several analogs of vitamin D under current investigation, is the compound's increased biological potency both *in vitro* and *in vivo*. Surprisingly, the biological potencies of many vitamin D compounds do not reflect their demonstrated biochemical affinities for the VDR (see Chapters 82–85). This is indeed true for 2MD, which,

despite its increased biological potency, binds to the VDR *in vitro* with an equilibrium dissociation constant almost equal to that of $1,25(OH)_2D_3$ [23]. One possible explanation for 2MD's increased potency *in vivo* as well as *in vitro* is the finding that 2MD binds weakly, if at all, to the vitamin D–binding protein (DBP), a serum macromolecule generally believed to transport vitamin D metabolites in the blood (see Chapter 8). This feature (lack of DBP binding) could facilitate the uptake of 2MD into target cells, thus increasing the analog's apparent potency by promoting elevated tissue localization. Indeed, this appears to be the case experimentally, as 2MD quickly disappears from the blood and rapidly localizes within tissues that include the cells of bone [26]. Lack of DBP binding, however, may also lead to an increase in the metabolism of 2MD, thereby reducing or altering its overall biological profile. Key *in vivo* studies do not appear to support this possibility, however, suggesting that perhaps 2MD is carried in the blood by other protein components. Despite these potential explanations for the enhanced potency of 2MD *in vivo*, this analog also stimulates the expression of a number of genes *in vitro* in cultured cells at very low concentrations, irrespective of serum DBP content. This finding suggests the possibility of a molecular/cellular component to explain the potency of 2MD.

The above studies prompted Yamamoto *et al*. [27] to investigate the mechanism of action of 2MD in osteoblastic cells *in vitro* and to contrast the analog's effects with those of $1,25(OH)_2D_3$. Initial studies explored the possibility that while the relative affinity of 2MD was identical to that observed for $1,25(OH)_2D_3$ in broken cell preparations, the binding of 2MD to the VDR might differ from that of $1,25(OH)_2D_3$ in intact living cells. Accordingly, osteoblasts were incubated with radiolabeled $1,25(OH)_2D_3$ or 2MD and the uptake of the two ligands and their association with the VDR was measured. Interestingly, both time dependent uptake as well as binding of each of the ligands to the VDR was similar, ruling out the possibility that the uptake of the two ligands into the intact cell was different, or that the relative affinities of $1,25(OH)_2D_3$ and 2MD were different. These results prompted a further comparison of the ability of the two ligands to induce accumulation of the VDR and its heterodimeric partner RXR on specific target genes in living cells using chromatin immunoprecipitation assays. Cells were incubated with increasing concentrations of either $1,25(OH)_2D_3$ or 2MD and the binding of both VDR and RXR to the vitamin D response elements (VDRE) located in the promoters of genes for both the 25 hydroxyvitamin D_3-24 hydroxylase (Cyp24) and osteopontin (OPN) was assessed. Unlike the results

seen in the ligand binding assays, however, 2MD is seen in Fig. 5 to display a two log increase in potency in its ability to promote VDR binding to each of the above gene promoters. Further studies revealed that the accumulation of both VDR and RXR on these gene
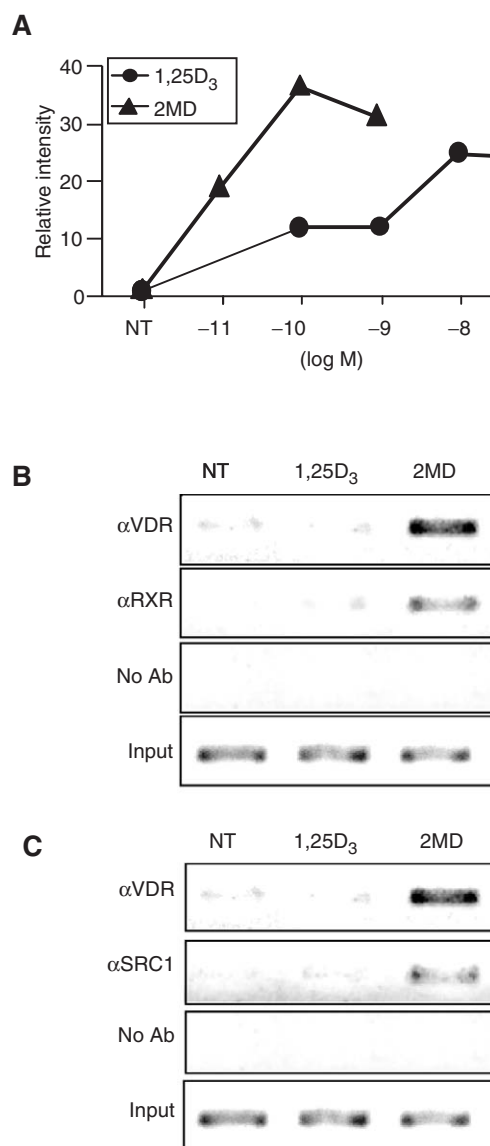
FIGURE 5   2MD potently stimulates binding of VDR to active promoters in intact cells. **A,** MC3T3-E1 cells were incubated under serum-free conditions for two hrs with increasing concentrations of $1,25(OH)_2D_3$ or 2MD and then subjected to chromatin immunoprecipitation (ChIP) analysis using anti-VDR or anti-RNA pol II antibodies. Isolated DNA was subjected to PCR using primers designed to amplify a 196 bp fragment of the OPN promoter from −854 to −658 that contained the VDRE at −764 to −748. The data represent a densitometric quantitation of the resulting PCR fragments. **B** and **C,** cells were treated with either vehicle, or 0.1 nM $1,25(OH)_2D_3$ or 2MD for 45 min. and then subjected to ChIP using antibodies to VDR, RXR, or SRC-1 as indicated. The resulting DNA was amplified as in **A,** and the PCR products are shown directly.

promoters also resulted in secondary consequences. Both SRC-1 and DRIP205, coactivators necessary for transcriptional activation of Cyp24 and OPN, were also recruited to the two genes in response to 2MD in a highly potent manner. Importantly, these data correspond directly with 2MD's ability to induce the expression of each of these genes in osteoblasts, and indeed other gene targets [27]. These findings suggest that despite similar pools of both 2MD- and $1,25(OH)_2D_3$-bound VDR, those receptors linked to 2MD display an increased capacity to localize on the regulatory elements present in the promoters for Cyp24 and OPN and to activate the expression of those genes.

These observations suggest the possibility that 2MD might induce an unusual conformation within the VDR that favors increased accumulation or increased retention of the receptor on VDREs. Can this unique conformational state induced by 2MD be detected *in vitro*? We evaluated this possibility with both X-ray diffraction studies using purified rat VDR [28] and by subjecting individual ligand receptor complexes to limited tryptic digestion *in vitro* [27]. Interestingly, while the digestion studies observed in Fig. 6 indicate that 2MD is able to promote a VDR conformation that is differentially proteolyzed relative to $1,25(OH)_2D_3$, this unique structure is not evident in the crystal structure of the rat VDR ligand binding domain in complex with either 2MD or $1,25(OH)_2D_3$. Thus, the overall special organization of this portion of the VDR is similar, irrespective of whether it is bound to $1,25(OH)_2D_3$ or 2MD. This finding is consistent with those of other investigators who have also failed to demonstrate a unique conformational difference within the ligand binding domain of the VDR via X-ray crystallography in the presence of superagonist analogs such as $(20S)-1,25(OH)_2D_3$ or KH1060 [29]. Nevertheless, these results again contrast with the positive results observed with such compounds in proteolytic digestion assays [30]. Thus, it is possible that visualization of these unique ligand-induced conformations at the atomic level will require three-dimensional structures of full-length VDR or cocrystallization of VDR with RXR or perhaps another of its interacting coactivator partners. Since synthetic ligands are known to alter the natural hormone-induced conformations of several different nuclear receptors [31], such discovery should come as no surprise when it is accomplished for the VDR.

VDR binding to most VDREs *in vitro* requires RXR, which forms an active heterodimer in response to $1,25(OH)_2D_3$ and participates with the VDR in direct DNA binding (see Chapters 11, 13, 18, and references therein). This requirement of the VDR for RXR in VDRE binding in intact cells has recently been
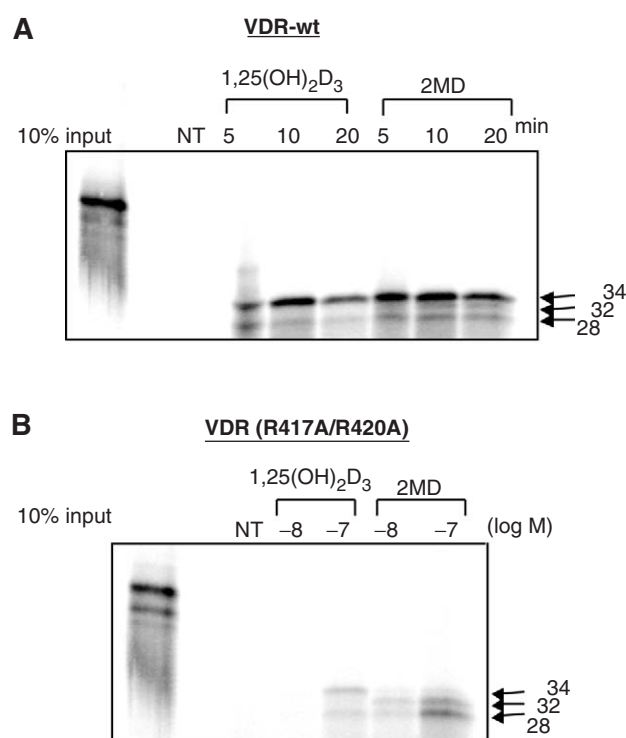
FIGURE 6   2MD induces a unique VDR conformation. [35S]-methionine-labeled hVDR or mutant hVDR(R417A/R420A) was incubated with the indicated concentrations of either $1,25(OH)_2D_3$ or 2MD and then treated with trypsin for the indicated time (**A**) or 10 min (**B**). Proteins were resolved using SDS-PAGE and autoradiographed. Wildtype hVDR, upper: hVDR(R417A/R420A), lower.

confirmed [27,32]. VDR also functions together with its heterodimer partner to recruit additional protein complexes that are required for the eventual activation of gene expression (see Chapters 11, 13–17, and references therein). These known particulars of VDR action prompted us to explore the possibility that the increase in affinity of the VDR for inducible promoters such as Cyp24 or OPN in response to 2MD might occur as a result of enhanced interaction with either RXR, with coactivators such as DRIP205 or SRC-1, or both. Surprisingly, the dose-dependent abilities of 2MD and $1,25(OH)_2D_3$ to induce a biochemical interaction between purified VDR and RXR, SRC-1 or DRIP were virtually identical *in vitro* [27]. This suggests that 2MD is incapable of promoting an enhanced interaction between the VDR and its various interacting partners. A completely different profile, however, was observed when these interactions were explored in intact cells using a mammalian two-hybrid system. In this system, the VDR was linked to the transactivation domain of VP16, and the VDR interacting domains of RXR, SRC-1, and DRIP205 were coupled to the DNA

A

B

C
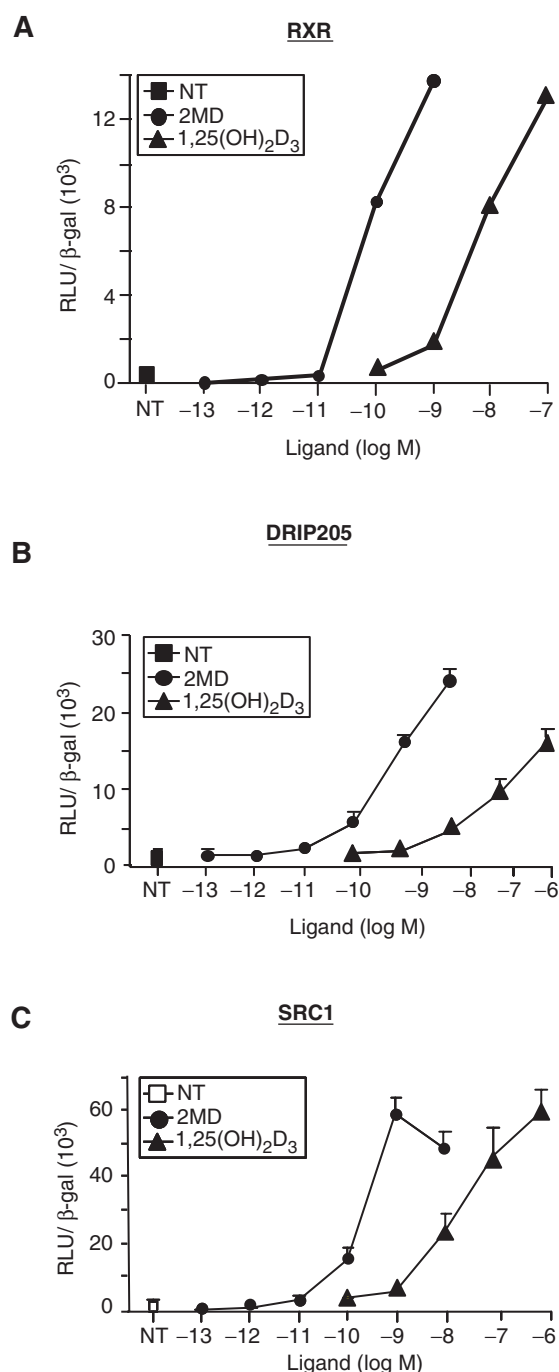
FIGURE 7 2MD is a potent inducer of VDR/RXR, VDR/SRC-1 and VDR/DRIP205 interactions assessed by yeast 2-hybrid assays 6. MC3T3-E1 cells were transfected with expression vectors for chimeric VDR-VP16 and chimeric RXRα- (**A**), SRC-1- (**B**) or DRIP205- (**C**) Gal4 DNA binding domain (DBD) fusions, and a multimerized Gal4 response element-luciferase reporter vector. Cells were treated with increasing concentrations of either $1,25(OH)_2D_3$ or 2MD and luciferase activity assessed after 24 hr.

binding domain of the yeast protein Gal4. Accordingly, 2MD was again discovered as documented in Fig. 7 to be at least two logs more potent in its ability to promote an interaction between VDR and either RXR, SRC-1 or DRIP205. These results support the idea that the increased potency of 2MD resides in its ability to promote both VDR DNA binding and complex formation on active gene promoters. Despite the lack of support for this hypothesis in the VDR crystal structure, it still seems likely that this effect of 2MD is driven through a unique ligand-induced conformation imposed on the VDR during complex formation.

The studies described above indicate that 2MD and perhaps other 2 carbon-modified $1,25(OH)_2D_3$ analogs are highly potent and potentially gene-selective activators of transcription in bone cells. This balance between its highly potent and potentially gene-selective action in bone and its $1,25(OH)_2D_3$-like activity in the gut makes it an excellent candidate for the therapeutic treatment of osteoporosis where normal intestinal absorption of calcium is required. As indicated above, however, while some understanding of the increased potency of 2MD and perhaps other analogs is emerging, additional work will be necessary to completely elucidate this facet of 2MD activity. Additional efforts will also be necessary to delineate its actions directly on the osteoblast to induce transcription in a gene- and perhaps cell-selective manner.

VIII. 2-METHYLENE-19-NOR-1α-HYDROXYPREGNACALCIFEROL (2MPREGNA), 2-METHYLENE-19-NOR-1α-HYDROXYHOMOPREGNACALCIFEROL (2MP) AND 2-METHYLENE-19-NOR-(20S)-1α-HYDROXYBISHOMOPREGNA-CALCIFEROL (2MBISP)

The compounds shown in Fig. 8 represent important new analogs that in the case of the bishomo compound 2MbisP and the pregnacalciferol compound 2Mpregna completely lack calcemic activity and have slight intestinal calcium transport activity in the case of the homo-pregnacalciferol compound (2MP). Note especially that these compounds completely lack the side chain structure of $1,25(OH)_2D_3$ and have no 25-hydroxyl group, possessing only a short hydrocarbon side chain. These compounds bind very well to the VDR; they are slightly less active in binding to the receptor than $1,25(OH)_2D_3$. They all possess activity in cellular differentiation, although not quite as effective as $1,25(OH)_2D_3$. The results shown in Fig. 9 demonstrate that these compounds

2-methylene-19-nor-1α-hydroxypregnacalciferol

2-Mpregna

2-methylene-19-nor-1α-hydroxyhomopregnacalciferol

2-MP

2-methylene-19-nor-(20S)-1α-hydroxybishomopregnacalciferol

2-MbisP
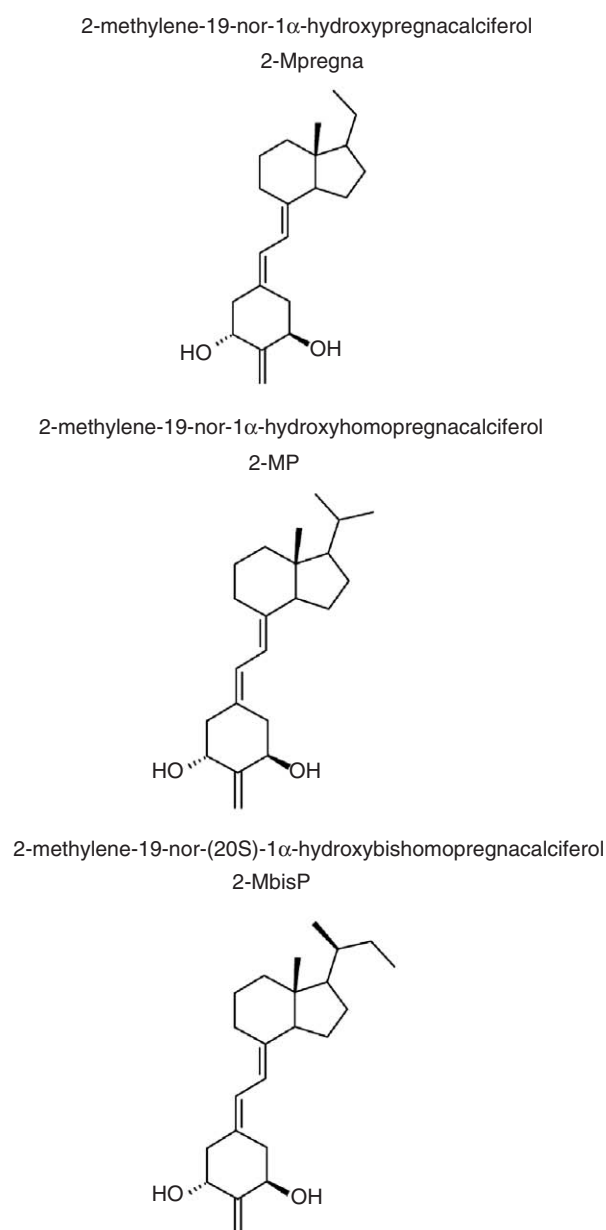
FIGURE 8   Structures of the noncalcemic 2-methylene-19-nor-1α-hydroxyvitamin D derivatives that possess a markedly shortened hydrocarbon side chain without a side chain hydroxyl.

are able to suppress serum PTH levels in intact animals at doses wherein serum calcium is not increased. In fact, PTH suppression is found at less than 1 µg/kg and not shown here, doses as high as 66 µg/kg will not increase serum calcium. Although these compounds are relatively new on the scene, we believe they have promise of being truly noncalcemic analogs that may be used for the treatment of conditions where a rise in serum calcium is not desirable. Such conditions are the treatment of autoimmune disease, oral treatment of psoriasis, and the treatment of malignancy.

IX. 2-METHYLENE-19-NOR-PREGNACALCIFEROL (2MPREGNA), 2-METHYLENE-19-NOR-1α-HYDROXYHOMOPREGNACALCIFEROL (2MP) AND 2-METHYLENE-19-NOR-(20S)-1α-HYDROXYBISHOMOPREGNA-CALCIFEROL (2MBISP): MOLECULAR MECHANISMS OF TISSUE SELECTIVITY

The most striking general feature of some but not all vitamin D analogs is their reduced ability or their apparent inability to induce hypercalcemia via normal physiological actions on the intestine, bone, and kidney while still retaining their ability to activate or suppress the expression of genes which may not be directly involved in calcium homeostasis. This feature stands in stark contrast to that of $1,25(OH)_2D_3$, and suggests that some analogs may exhibit tissue- or gene-selective activities. Clearly, analogs with this type of biological profile would be attractive for such therapeutic indications as autoimmune disease, psoriasis, or cancer, where an increase in serum calcium levels is not desirable and most likely detrimental. The 2-carbon-modified homopregnacalciferols appear to be such compounds. Accordingly, while preliminary studies (Plum and DeLuca, unpublished) indicate that they may promote cancer cell differentiation *in vitro* and are highly effective in suppressing serum PTH levels *in vivo*, as seen in Fig. 9, presumably by inhibiting the synthesis/secretion of PTH from the parathyroid glands, they are correspondingly unable to induce hypercalcemia in normal animals.

The underlying mechanism of this feature of these modified pregnacalciferols, as well as other vitamin D analogs with similar properties, is largely unknown. The general biochemical characteristics of these compounds relative to $1,25(OH)_2D_3$ include a similarity in affinity for the VDR relative to $1,25(OH)_2D_3$ and at least in cultured cells *in vitro*, a similarity in biopotency. The 2-carbon modified homopregnacalciferols are clearly not superagonists, however, and thus do not exhibit properties of increased potency such as those manifested by compounds such as 2MD.

As with the 2 carbon-modified analogs of 19-nor-$1,25(OH)_2D_3$ discussed earlier, the fundamental unanswered question with respect to the 2-carbon modified homopregnacalciferols is whether the apparent tissue selectivity seen *in vivo* is a function of unique pharmacokinetic properties that are different from those of $1,25(OH)_2D_3$ or whether this selectivity is manifested at the level of the target cell. A significant limitation in answering this question resides
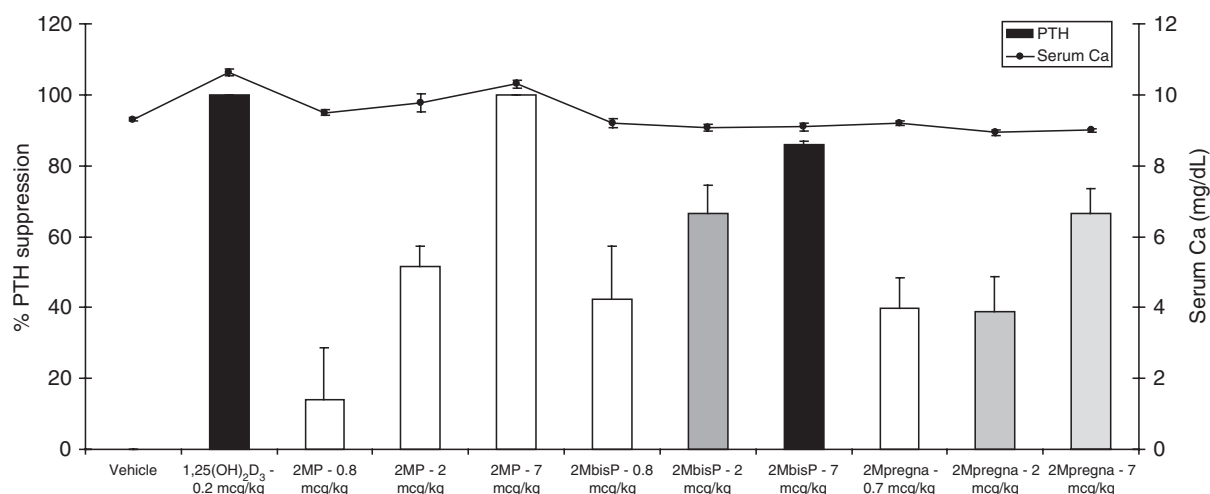
FIGURE 9   Biological potency of 2-MP, 2-MbisP, and 2-Mpregna *in vivo*. Adult female Sprague Dawley rats (6/group) were fed a 0.47% calcium, 0.3% phosphorus purified diet for one wk. At this point, the animals were given the dose indicated i.p. for seven consecutive days except $1,25(OH)_2D_3$, which was given only for four days. All animals were continued on the 0.47% calcium, 0.3% phosphorus purified diet. Twenty-four hr after the last injection, animals were sacrificed and serum calcium determined by atomic absorption spectrometry. Serum PTH was measured using an ELISA assay (Immutopics, Inc.). The vertical bars represent standard errors of the mean.

both in the gaps that are currently present in our understanding of how calcium is absorbed from the intestine or resorbed from bone, as well as the limitations present in our understanding of the mechanisms by which $1,25(OH)_2D_3$ regulates those processes (see Chapters 24, 25, and 38). Nevertheless, significant progress is being made in delineating both processes, and has revealed the central importance of both the intestinal epithelial calcium channel proteins (ECAC1 and 2) and calbindin 9K in the absorption of calcium from the gut, and osteoblast-produced receptor activator of NF-κB ligand (RANKL) as a signaling mediator of bone calcium resorption via the osteoclast. Importantly, $1,25(OH)_2D_3$ regulates the expression of both genes. Accordingly, we explored the ability of the modified homopregnacalciferols to induced ECAC2 and calbindin 9K in the $CaCO_2$ intestinal cell model and RANKL in the MC 3T3-E1 osteoblast cell model. Interestingly, both $1,25(OH)_2D_3$ and 2MbisP are almost equipotent in their capacity to stimulate the expression of the three genes in their respective cell backgrounds (Watanuki *et al.*, unpublished). With respect to intestinal calcium absorption, however, the failure of 2MbisP to support the transcription of other factors that may be essential to the calcium transport process could still result in the *in vivo* phenotype of insensitivity to the 2MbisP. Clearly, much more needs to be done before a clearer understanding of the underlying mechanism emerges.

X. SUMMARY

The development of several important new analogs of $1,25(OH)_2D_3$ are described wherein the 19-carbon is deleted and replaced by 2 hydrogens. The configuration of carbon-20 has been altered to the S-configuration, and carbon-2 has substituted either a 2-methylene or a 2α-methyl group. The resulting compounds are 2-methylene-19-nor-(20S)-1α,25(OH)₂D₃ (2MD) and 2α-methyl-19-nor-(20S)-1α,25(OH)₂D₃ (2AMD) and show bone calcium mobilizing activity 30 times that of the native hormone while showing equal activity in intestinal calcium transport and binding to the VDR. 2MD specifically stimulates osteoblast cultures to form new bone, a property minimally expressed in the case of $1,25(OH)_2D_3$. Further, 2MD markedly increases bone mass of ovariectomized aged female rats while $1,25(OH)_2D_3$ has minimal but significant activity. 2MD is currently being developed as an anabolic bone agent for the treatment of bone loss diseases such as osteoporosis.

By replacing the side chain of 2MD with hydrocarbon side chains of 2–4 carbons without an hydroxyl group, true noncalcemic analogs have been produced. These compounds bind to the receptor almost as well as the native hormone, have significant cellular differentiation activity but lack the ability to raise serum calcium concentration either from bone or from intestine. These compounds are currently being studied for the

treatment of disease where an elevation of calcium is undesirable. *In vivo* these compounds suppress the parathyroid gland while having no effect on serum calcium concentration.

All of the above modified forms of the vitamin D hormone are fully active in transcriptional assays using the reporter gene attached to the 24-hydroxylase promoter and current assays suggest that 2MD causes the formation of a stable complex of VDR to VDREs and markedly improves the affinity of that complex for these response elements. Beyond this, little is known concerning the mechanism whereby 2-methylene-19-nor-(20S)-1,25(OH)$_2$D$_3$ is not only more potent but much more selective for activity in bone than is 1,25(OH)$_2$D$_3$.

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Nonsteroidal Analogs

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I. INTRODUCTION

Vitamin D is a steroid hormone identified originally as a preventative of the skeletal disease rickets (reviewed in [1]). In addition to key functions in bone health and development, vitamin D has been shown to play a role in regulating differentiation and proliferation of various cell types. The biologically active component of vitamin D is $1\alpha,25$-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which is synthesized through a series of tightly regulated metabolic steps in the liver and kidney (see Chapters 1–5) and binds to the nuclear hormone vitamin D receptor (VDR) to activate gene expression (see Chapters 11–22). $1,25(OH)_2D_3$ functions to control calcium and phosphate levels through regulation of intestinal absorption of calcium and phosphate, reabsorption of calcium in the kidney, and mobilization of calcium from bone [2]. Circulating $1,25(OH)_2D_3$ is bound to vitamin D binding protein (DBP), which may serve to transport it to sites of action and to extend its serum half-life (see Chapters 8 and 9). Additional regulation of $1,25(OH)_2D_3$ activity occurs through a feedback loop that senses an increased level of $1,25(OH)_2D_3$ and induces its own degradation via up-regulation of the enzyme 24-hydroxylase (24-OHase) (see Chapters 2 and 6). In the case of greater than physiological levels of vitamin D activity, excess calcium is absorbed via the intestine leading to hypercalcemia, which is the main side effect that limits the use of current vitamin D analogs in many disease indications such as cancer and osteoporosis. The development of vitamin D analogs that exhibit improved therapeutic efficacies stem from analogs that have a reduced binding to DBP, altered metabolic properties, improved pharmacodynamic profiles, and induction of unique receptor conformations [3,4] and Chapters 82 and 83. All but a few of the compounds generated to date are secosteroid analogs that are based on the vitamin D structure (Fig. 1).

This chapter will highlight the development of small molecule vitamin D_3 mimics whose design is based on the premise that they manifest properties inherently different from that of the endogenous hormone or its secosteroid derivatives. These include minimal binding to DBP and reduced metabolism by the 24-hydroxylase. The unique structural features of these compounds that distinguish them from vitamin D secosteroid derivatives highlight the possibility that they will exhibit novel pharmacokinetic and pharmacodynamic profiles, which can be manipulated to create molecules with less calcemic effects than classic vitamin D analogs. This rationale is based on the well-established fact that synthetic ligands for nuclear hormone receptors can induce unique conformations in the receptor that result in altered tissue and gene specificity as compared to the endogenous steroid ligands [5,6]. Examples of this include tamoxifen and raloxifene, which are tissue-selective modulators of estrogen receptor activity. There are very few reports of nonsecosteroidal vitamin D mimics reported in the literature, and most of the effort has been directed towards side-chain secosteroid analogs derived from vitamin D_3. There are three classes of compounds with biological data published that may be considered nonsecosteroid vitamin D receptor agonists. These include nonsteroidal molecules based on a bisphenol scaffold (i.e., LG190119) [7], molecules in which the C and/or D ring of $1,25(OH)_2D_3$ have been removed [8,9], and the secondary bile acid lithocholic acid (reviewed in Chapter 53; [10]) (see Fig. 2). A review of the structures and activity of the bisphenol and C/D ring modification class of compounds is included below.

II. NONSECOSTEROID BISPHENOL COMPOUNDS

A. Identification, Structure, and Synthesis

Based on clinical success with estrogen receptor modulators, the identification of synthetic ligands for
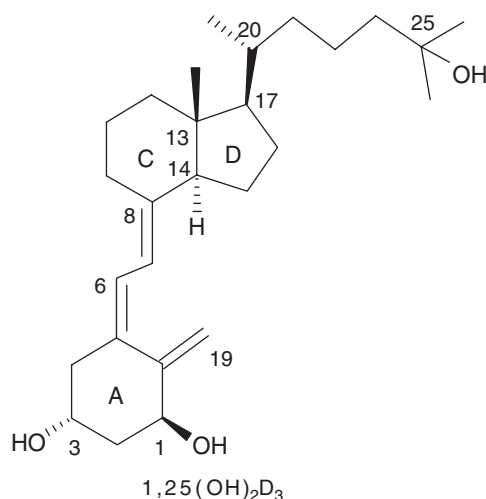
FIGURE 1 Structure of 1,25(OH)$_2$D$_3$. Schematic illustration of the 1,25(OH)$_2$D$_3$ molecule with the A-, C-, and D-rings marked, and carbon residues numbered for reference. The side chain referred to in the text and modified in many analogs to alter DBP binding is off of C17.

nuclear hormone receptors that mimic the activity of the endogenous hormone, while reducing their unwanted physiological side effects has been the therapeutic target for many drug discovery nuclear receptor programs. The bisphenol structure template was identified through a cell based high-throughput screen of small molecule compound libraries in which VDR was cotransfected with a reporter containing a VDR response element from the 24(OH)ase promoter [7]. The original screening hit was optimized for potency (40 nM) and efficacy (90%) to approach the activity of 1,25(OH)$_2$D$_3$ (2–5 nM; 100%). The bisphenols are to date the only VDR ligands with associated *in vitro* and *in vivo* published data that are not derived from the 1,25(OH)$_2$D$_3$ core structural scaffold. The characteristics of these compounds are reviewed below.

B. *In Vitro* Characterization of Bisphenol Analogs

The original screening hit, LG190090, was a bisphenol derivative with an EC$_{50}$ of 2.2 μM and efficacy of 80 percent compared to 1,25(OH)$_2$D$_3$ (see Table I). The series of published structures indicate a significant improvement in potency (LG190178 at 40 nM) was obtained by modifications including addition of a diethyl group to the bridgehead of LG190090 and hydroxyl group substitution of one of the 3,3-dimethyl-2-butanone groups. These compounds were shown to require VDR for activation and did not cross react with other nuclear hormone receptors. The more potent
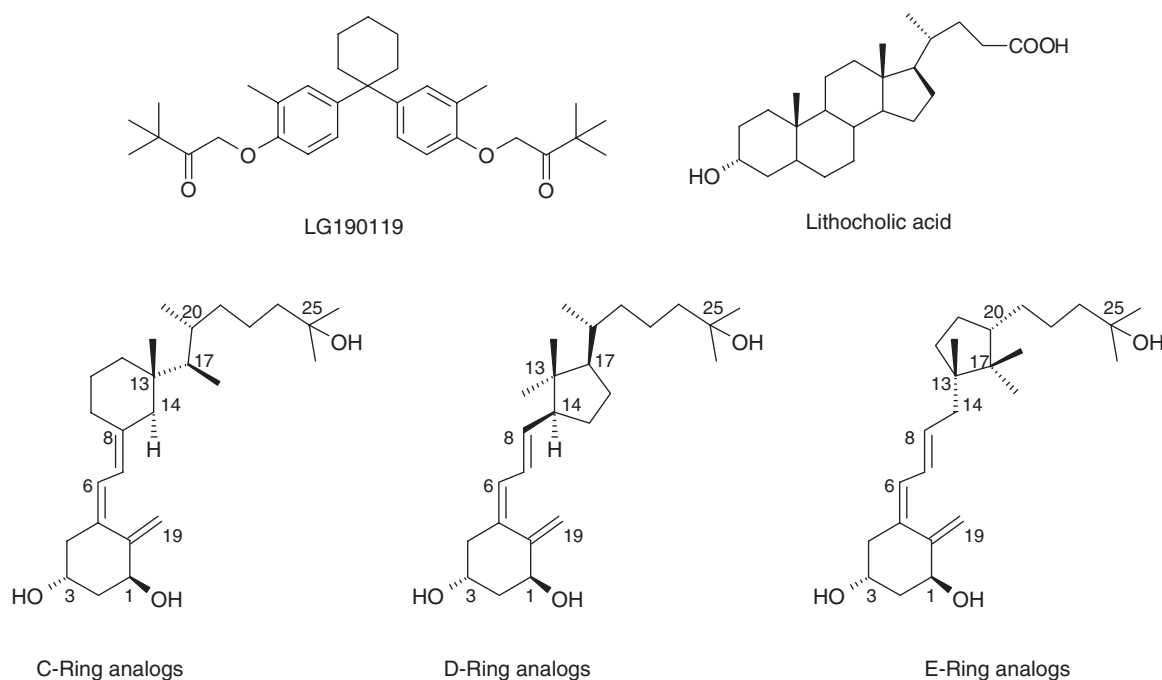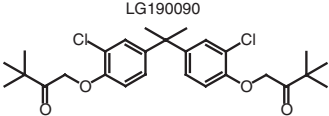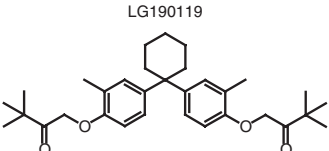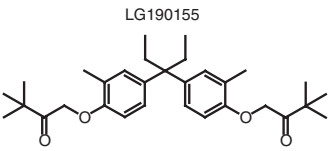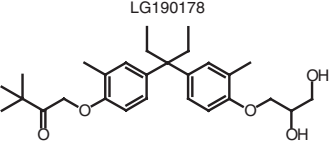
FIGURE 2 Structures of various nonsecosteroid vitamin D receptor ligands. The three types of nonsecosteroid molecules are represented by selected structures, including the bisphenols (LG190119), bile acids (lithocholic acid), and C/D ring modifications that disrupt the core secosteroid scaffold or introduce novel structural motifs (E-ring analogs).

TABLE I   Structures and Activities of Selected Bisphenol Nonsecosteriod Compounds

Compound	CTF[1] EC$_{50}$ (nM)	VDR binding[2] Ki (nM)	LNCaP[3] EC$_{50}$ (nM)	HL60[4] EC$_{50}$ (nM)	NHEK[5] EC$_{50}$ (nM)	DBP binding[6] IC$_{50}$ (nM)
1,25(OH)$_2$D$_3$	2–5	0.5	2	8–10	10–30	200(h); 40(r)
LG190090	2500	>10000	3000	10000	ND[7]	>10000
LG190119	2200	>10000	2000	2000	500	>10000
LG190155	600	>10000	300	800	500	>10000
LG190178	40	150	20	30–50	30–50	>10000

[1]Cotransfection (CTF) data was obtained using pRShVDR and VDRE(1)-ΔMTV-Luc in HepG2 cells.
[2]VDR binding affinity was determined by competition binding with yeast expressed hVDR and [^{3}H]-1,25(OH)$_2$D$_3$.
[3]Potency of LNCaP growth inhibition determined by BrdU incorporation.
[4]Potency of HL60 differentiation determined by nitroblue tetrazolium assay.
[5]Potency of normal human epidermal keratinocytes (NHEK) growth inhibition determined by BrdU incorporation.
[6]Human (h) and rat (r) DBP binding determined by competition binding versus [^{3}H]-1,25(OH)$_2$D$_3$.
[7]ND is not determined.

compounds were shown to bind directly to VDR through ligand-binding assays in which ^{3}[H]-1,25(OH)$_2$D$_3$ was displaced by an unlabeled competitor. Interestingly, the binding affinity of the compounds was less than that predicted by the cell-based functional assays, which may be attributed to a slow off-rate of 1,25(OH)$_2$D$_3$.

Several cancer cell lines including prostate [11], breast [12,13], and colon cells [14] have been shown to respond to the antiproliferative activity of 1,25(OH)$_2$D$_3$, which is a consequence of cell cycle arrest [13,15] and in some cases programmed cell death [16]. Activity in cell lines is a significant marker that has served as the basis for further study of vitamin D analogs in animal tumor models. More potent antiproliferative activity relative to 1,25(OH)$_2$D$_3$ in these cell lines has been a prime objective in the design of improved vitamin D analogs in order to maximize their efficacy *in vivo*. Results from testing of the bisphenol compounds

indicate that they retain the antiproliferative activity that would be expected from a VDR agonist [7]. LG190178 inhibited proliferation of human SK-BR-3 breast cancer cells and LNCaP prostate cancer cells at concentrations ranging from 30–50 nM. The antiproliferative potency correlates well with the activity of the compounds in the VDR cotransfection assay (Table I). Further studies demonstrated these compounds could efficiently differentiate HL60 human leukemia cells into macrophages with the most potent compound, LG190178, having an EC$_{50}$ value of 30 nM (Table I). Another important and well-established role for 1,25(OH)$_2$D$_3$ is the control of keratinocyte proliferation and differentiation [17,18]. To determine if the nonsecosteroid compounds could replicate this aspect of 1,25(OH)$_2$D$_3$ activity, compounds were added to human epidermal keratinocytes and cultured for seven days. Treatment with selected LG compounds resulted in growth inhibition and potencies

that were consistent with the rank order of the compounds in both cotransfection experiments and inhibition of proliferation in other cell types such as LNCaP and HL60. As in the other experiments, the most potent compound was LG190178. The morphology of the treated keratinocytes was also shown to be similar to that reported for $1,25(OH)_2D_3$ [19], supporting the fact that synthetic nonsecosteroid molecules working through the VDR can mimic vitamin D activities.

$1,25(OH)_2D_3$ exposure to target tissues has been shown to partly depend on binding to DBP, which controls delivery to site of action and serum half-life [3,20]. Binding to DBP is therefore a major influence on the pharmacokinetic and pharmacodynamic properties of the vitamin D analogs. Structural evaluation of $1,25(OH)_2D_3$ indicates that the regions of the molecule contributing to DBP binding include the C-17 side chain and the D-ring. A correlation has been established between diminished DBP binding and reduced calcemic activity, although there are exceptions such as the analog ED71, which appears to have enhanced DBP binding but reduced calcemic activity [21]. Much of the chemistry on secosteroids has focused on modifications to the C-17 side chain region of the molecule in order to reduce DBP binding. Advanced clinical leads such as EB1089 (Fig. 3 and Chapter 84) display significantly less binding to DBP compared to $1,25(OH)_2D_3$. One prediction of nonsecosteroids is significantly reduced DBP binding, and binding studies with the bisphenol compounds support this rationale, as none of the compounds tested displayed any detectable DBP binding (Table I) [7].

C. *In vivo* Characterization of Bisphenol Analogs

Many vitamin D secosteroid analogs exhibit promising *in vitro* profiles, but *in vivo* retain their ability
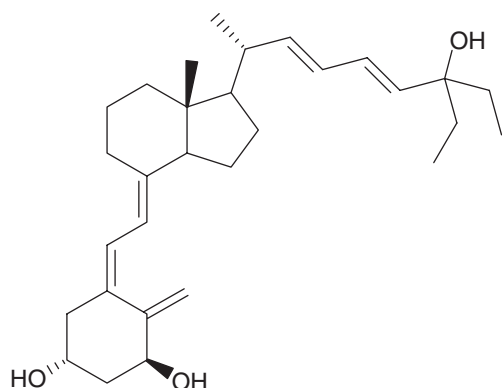
FIGURE 3   Structure of EB1089. Schematic illustration of the vitamin D_3 analog EB1089 (Seocalcitol).

to induce hypercalcemia. The bisphenol Vitamin D mimics were therefore assayed *in vivo* to determine their ability to induce VDR target genes and the potential for separation of calcemic activity from transcriptional activity. In the first report [7], LG190119 (10 mg/kg and 30 mg/kg) was administered orally to BALB/c mice and the serum calcium and kidney 24(OH)ase RNA levels were measured. In this study, LG190119 induced kidney 24-OHase expression approximately tenfold at a dose of 30 mg/kg, administered daily for 3–5 days by oral gavage without a significant increase in serum calcium. These results were confirmed in a second study in which (10 mg/kg; 5 day p.o.), LG190119 gave a 30-fold increase in 24(OH)ase levels with no significant increase in serum calcium. In the same experiment, $1,25(OH)_2D_3$ induced 24(OH)ase 25-fold (5 µg/kg; 5 days p.o.), but significantly increased serum calcium. The most potent compound, LG190178, was not included in these studies. The results demonstrate that based on the comparison of induction of 24(OH)ase mRNA levels to plasma calcium levels, the nonsecosteroid bisphenol compounds may possess an improved therapeutic index. This conclusion assumes that 24(OH)ase mRNA serves as a suitable biomarker for VDR activity, and indicates a separation of VDR target gene expression from calcium induction.

Further work has examined the ability to translate the antiproliferative activity of these compounds on various cancer cell lines *in vitro* to *in vivo* effect in tumor models. These studies [22] were carried out using LG190119 in a prostate cancer model in which LNCaP xenograft tumors are established in athymic mice. Treatment with LG190119 (dosed every other day at 10 mg/kg over a 12 week period) inhibited the growth of these tumors (as measured by tumor volume) without significantly increasing serum calcium levels. The activity of LG190119 was compared to $1,25(OH)_2D_3$ and EB1089, a less calcemic vitamin D analog currently in clinical trials for treatment of various cancers and shown to be threefold more potent in inhibiting LNCaP cell growth than $1,25(OH)_2D_3$ [15,23]. The efficacy results with LG190119 compared favorably to EB1089, which although inhibiting tumor growth, in these studies was hypercalcemic (dosed at 1 µg/kg), and $1,25(OH)_2D_3$ which in these studies did not have an effect on tumor growth at the dose tested (0.5 µg/kg).

In summary, the nonsecosteroid bisphenol template has been shown to bind to and transactivate VDR, activate VDR target genes, and inhibit growth of cancer cells. Animal studies with these compounds demonstrate separation of gene induction and calcium increase indicating that a nonsecosteroidal core with inherently unique pharmacologic and molecular properties may translate into vitamin D mimics with

III. BISPHENOL ANALOGS AS SELECTIVE AGONISTS OF MUTANT VDR

Hereditary vitamin D resistant rickets (HVDRR) is an autosomal recessive disease characterized by vitamin D resistance in target organs even in the presence of high levels of circulating $1,25(OH)_2D_3$ (see Chapter 72 for a detailed review). The resistance to vitamin D was shown to result from mutations affecting expression and function of VDR. Several missense mutations have been described in the LBD of VDR that affect the binding affinity of $1,25(OH)_2D_3$ leading to impaired transcription of target genes. In a series of elegant experiments [24], Koh and co-workers have applied molecular modeling and structural based rational design to develop vitamin D mimics that would be complementary to the R274L mutation in the VDR ligand-binding domain and activate the mutant receptor efficiently. In addition to further validating this approach for ligand identification, compounds that could activate the mutant receptor with minimal side effects could potentially be useful as treatments for these patients.

The options for a starting template for the identification of analogs that could activate mutant VDRs included derivatives of $1,25(OH)_2D_3$ or selecting a nonsecosteroid molecule. The bisphenol scaffold was chosen for several reasons, including reduced calcemic activity compared to $1,25(OH)_2D_3$, and the ease of synthesis of multiple derivatives. Based on the crystal structure of $1,25(OH)_2D_3$ with VDR, computer modeling predicted that the nonsecosteroid LG190155 would fill the ligand-binding pocket such that one of the 3,3-dimethyl-2-butanone groups is able to hydrogen bond to His305, the amino acid residue contacted by the C22-C25 side chain of $1,25(OH)_2D_3$. This orientation of LG190155 allows the carbonyl oxygen of the other 3,3-dimethyl-2-butanone group to hydrogen bind to the Ser278 and Tyr143 side chains. These amino acid side chains are bound by the 3β-hydroxy group of $1,25(OH)_2D_3$. Computer modeling of LG190155 further predicted that unlike $1,25(OH)_2D_3$, the molecule would not interact directly with Arg274, the amino acid contacted by the 1α-OH of $1,25(OH)_2D_3$. This prediction was supported by functional assays indicating that LG190155 could activate the R274L mutant VDR with approximately the same potency and efficacy as wild-type VDR.

Through further use of computer-aided rational design, the nonsecosteroid scaffold was modified such that one of the 3,3-dimethyl-2-butanone groups was substituted with a limited set of alkylating agents. The model of LG190155 bound to the pocket allowed for the selection of 40 potentially useful substitutions, which after additional computational assessment were narrowed down to 13 optimal analogs. This approach to ligand identification was shown to be successful as 7 of the 13 compounds had activity on the R274L VDR better than LG190155 in terms of potency in cotransfection assays with the best compound, A-13 having EC_{50} of 3.3nM (Fig. 4).

As mentioned previously, the bisphenol nonsecosteroid scaffold was chosen for these studies based on ease of synthesis and less calcemic activity as compared to $1,25(OH)_2D_3$. The analogs optimized for activation of R274L were tested for their ability to stimulate Ca^{2+} influx in preosteoblastic cells. The results indicated that

LG190155
wtVDR EC_{50} 110 nM
mutVDR EC_{50} 85 nM

A-13 Koh
mutVDR EC_{50} 3.3 nM
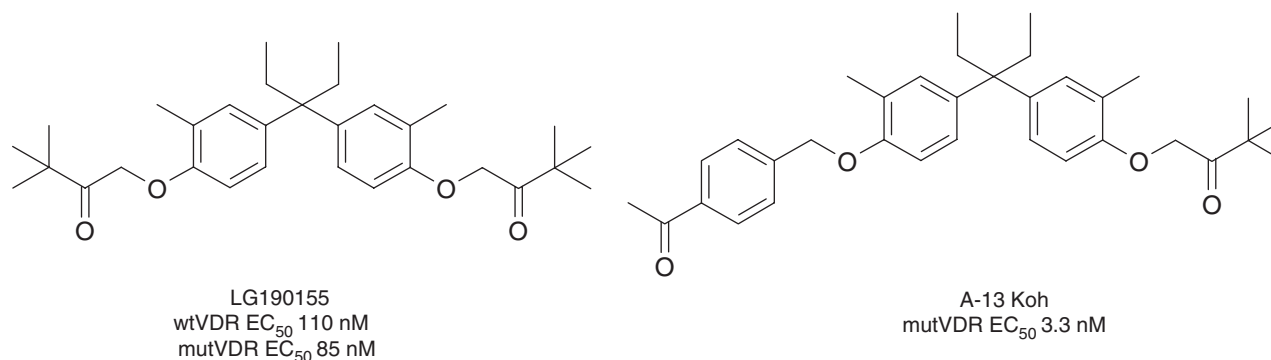
FIGURE 4   Structures of LG190155 and A-13. The structure and activity of the most potent analog, A-13, on the R274L mutant VDR is shown. The parent compound, LG190155, on the wild-type and R274L mutant VDR is also included for reference. The EC_{50}s were determined by transient cotransfection of HEK293 cells with a luciferase reporter plasmid with wild-type or R274L mutant VDR and treatment with compound for 36 hours. It should be noted, that the value of LG190155 is different from that in Table I (110 nM vs. 600 nM), which may be due to differences in the assay format including cell type and response element used in the cotransfection experiments.

while $1,25(OH)_2D_3$ stimulated Ca^{2+} by 50%, the analogs did not show any significant Ca^{2+} influx activity. While these data remain to be verified *in vivo*, they highlight the promise of using a nonsecosteroid VDR agonist that can be quickly and easily optimized, through computer aided drug design, to mutant forms of the receptor while retaining an intrinsically low calcemic activity shown previously for this molecular template.

IV. NON-SECOSTEROID CD RING MODIFICATIONS

One approach to identifying novel molecules has been to randomly screen small molecule libraries with a variety of cellular and biochemical based assays. In the case of VDR, the bisphenols are the only published structural class found to date using this approach (see Section II). An alternative approach has been to make modifications of the existing natural ligands that retain the desired biological activities while minimizing side effects. A large number of analogs of the vitamin D molecule have been synthesized through this approach, with relatively few demonstrating improved properties compared to the parent $1,25(OH)_2D_3$. A more radical form of structural modification to the core ring structure of the $1,25(OH)_2D_3$ molecule was carried out to identify regions of the molecule that could be further modified to optimize therapeutic activity. These modifications to the C- and D-rings disrupt the secosteroid structure, and are reviewed here along with the generation of a new structural component referred to as an E-ring [8,9].

A. C- and D-ring Analogs

Modifications to the core secosteroid structure were made to explore the role of the C- and D-rings in the maintenance of proper conformation of the A-ring and the C-17 side chain of the vitamin D molecule. Interestingly, the work from Verstuyf *et al.* [8,9] identified a series of potent and efficacious nonsecosteroidal VDR ligands that lacked either the C- or D-ring in certain cases or introduced a novel 5 membered "E"-ring as a substitution for the C/D rings (Fig. 5).

In order to generate these compounds, an acyclic parent compound (KS 018) was made which when tested did not have any VDR activity or binding to DBP. Re-introduction of only the C-ring with the $1,25(OH)_2D_3$ side chain recovered most of the VDR binding activity for most of the analogs reported (see Table II; ZG1368 and CY625) as compared to $1,25(OH)_2D_3$, while maintaining low to no DBP binding.

In the cell lines tested, the antiproliferative activity of C-ring analogs was generally increased with some compounds being 30–60 fold greater than $1,25(OH)_2D_3$ (ZG1310; ZG1441; ZG1423). The serum calcium induction by the C-ring analogs was reduced by 100-fold for some of the active compounds as compared with $1,25(OH)_2D_3$. Additional modifications to these compounds led to a reduced VDR binding affinity (i.e., 19-nor-A ring), or increased antiproliferative activity (i.e., movement of 25-OH group to C-24(R); C-16 to C-17 double bond; C-23 to C-24 triple bond).

In order to evaluate further the structural requirements of the C- and D-ring in the core vitamin D secosteroid template, the D-ring alone was reintroduced into the acyclic backbone molecule (Table III). As with the C-ring analogs, the nonsteroidal D-ring compounds had greatly reduced binding to DBP (<10% of $1,25(OH)_2D_3$), although they retained significant VDR affinity (40–80% of $1,25(OH)_2D_3$). The D-ring analogs were generally less potent in antiproliferative assays compared to similar C-ring molecules, except for WU515 (Table III) and WU507 in which fluorination of C-26 and C-27 led to increased activity that was 10–50 percent more potent than $1,25(OH)_2D_3$ depending on cell type.

B. E-ring Analogs

Through the exploration of the structural requirements of the C- and D-rings, it was found that placement of a novel 5 member ring linking C13 to C20 could substitute for C- and D-rings and retain some vitamin D activity (Table IV). Referred to as an E-ring, this structural motif is apparently sufficient to introduce the proper spacing and conformation restraints on the A/secoB domains and side chain of $1,25(OH)_2D_3$. Analogous to the structural changes introduced in the C- and D-ring analogs, the most potent E-ring molecules were generated by modifications to the side chain including fluorination of C26 and C27 (CD 503; CD 504; CD 483) or elongation at the C26, C27, and C24 positions. The most active compounds, including CD 503, CD 504, and CD483, had 2–8 times greater antiproliferative activity and less than 5 percent the calcemic activity of $1,25(OH)_2D_3$. Interestingly, introduction of double and triple bonds between C23 and C24 reduced VDR binding affinity to 10% of $1,25(OH)_2D_3$ (compare CD 504 and CD 483 to KS 176), while retaining twice as much antiproliferative activity in MCF-7 cells. The effects of C26,C27 hexafluorination and unsaturation of the side chain in the E-ring analogs are consistent with the effects of the same
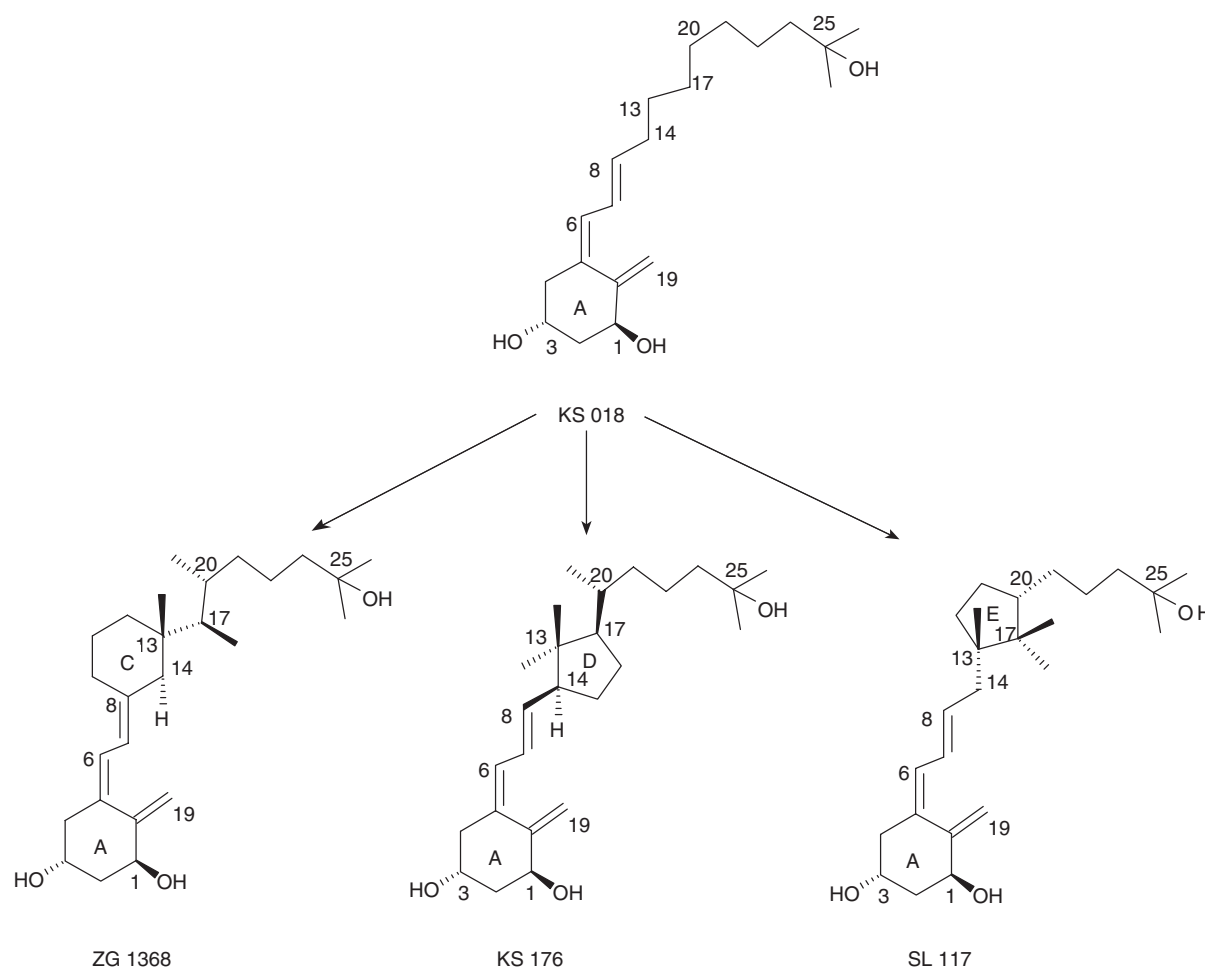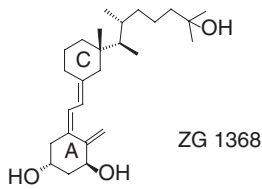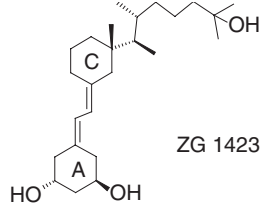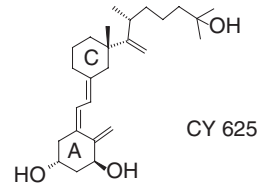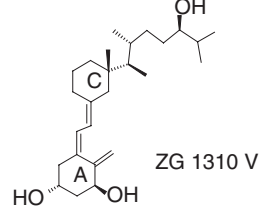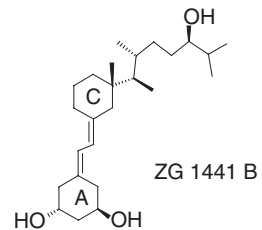
FIGURE 5   Identification of C-, D-, and E-ring modifications of 1,25(OH)₂D₃. The 1,25(OH)**₂**D**₃** molecule was reduced to a backbone structure that contained the A-ring and C17 side chain (KS018). The C- and D-rings were reintroduced separately to determine their relative contribution to the conformation and positioning of the A-ring and C-17 side chain. Representative molecules ZG 1368 (C-ring), and KS176 (D-ring) are shown. Also shown is SL 117, a representative of E-ring substituted molecules in which a novel 5-membered ring replaces the C/D ring of 1,25(OH)**₂**D**₃**.

modifications made to 1,25(OH)₂D₃. The antiproliferative activity of the E-ring analogs, combined with a relative lack of calcemic activity, provides these compounds with a potentially improved therapeutic profile over most secosteroid analogs, particularly in the treatment of various cancer types. It will be important to determine if these compounds retain their potent *in vitro* antiproliferative activities when tested *in vivo* and what effect the ring modifications may have on the pharmacodynamic and metabolic properties of these compounds.

The approach described above complements well the search for nonsecosteroidal vitamin D mimics in that fundamental changes to the core structure that generate nonsteroidal molecules result in improved

ratios of antiproliferative effects to calcemic activity. Verstuyf *et al*. [8,9] investigated the underlying bases for why and how the newly generated C/D ring and E-ring analogs could maintain vitamin D-like activity while minimizing their calcemic potential. The poor binding of all of the analogs to DBP can explain in part the reduced calcemic activity, as all but a few of the vitamin D analogs made to date that exhibit some separation of differentiation and calcemic activity also bind poorly to DBP. As discussed previously, DBP binding contributes to the serum half-life and tissue exposure of vitamin D analogs. Other factors examined included metabolism, receptor affinity, and induction of altered receptor conformations. Only the latter demonstrated a strong correlation with improved

TABLE II   Structures and Activities of Selected C-ring Modifications

Compound	VDR binding[1] (%)	DBP binding[2] (%)	HL60[3] (%)	MCF-7[4] (%)	Kerat[5] (%)	Serum Ca$^{+2;6}$ (%)
1,25(OH)$_2$D$_3$	100	100	100	100	100	100
ZG 1368	60	20	1000	6000	6000	50
ZG 1423	45	3	1000	2000	5000	13
CY 625	80	5	150	200	200	1
ZG 1310 V	70	10	3500	6000	6000	35
ZG 1441 B	35	2	3000	1000	3750	34

[1]Relative percent VDR binding affinity compared to 1,25(OH)$_2$D$_3$ (100%; 0.13 nM) was determined as the concentration of compound needed to displace 50% of [^{3}H]-1,25(OH)$_2$D$_3$ from pig intestinal mucosa expressed VDR.

[2]Relative percent affinity to hDBP (1,25(OH)$_2$D$_3$ at 100%; 220 nM) determined by competition binding versus [^{3}H]-1,25(OH)$_2$D$_3$.

[3]Relative percent activity of HL60 differentiation measured by nitroblue tetrazolium assay with 100% (29 nM) indicating the EC50 of 1,25(OH)$_2$D$_3$.

[4]Relative percent activity of MCF-7 growth inhibition determined by [^{3}H]thymidine incorporation with 100% (37 nM) indicating the EC50 of 1,25(OH)$_2$D$_3$.

[5]Relative percent activity of keratinocyte growth inhibition determined by [^{3}H]thymidine incorporation with 100% (5.3 nM) indicating the EC50 of 1,25(OH)$_2$D$_3$.

[6]Relative percent of serum calcium levels in mice after compound treatment with 100% (14.7 mg/dl) indicating 1,25(OH)$_2$D$_3$ at 0.4 µg/kg/day for 7 days.

TABLE III   Structures and Activities of Selected D-ring Modifications

Compound	VDR binding[1] (%)	DBP binding[2] (%)	HL60[3] (%)	MCF-7[4] (%)	Kerat[5] (%)	Serum $Ca^{+2;6}$ (%)
$1,25(OH)_2D_3$	100	100	100	100	100	100
SL 117	80	10	85	85	90	0.3
WU 515	70	3	1000	5000	3000	6

[1]Relative percent VDR binding affinity compared to $1,25(OH)_2D_3$ (100%; 0.13 nM) was determined as the concentration of compound needed to displace 50% of $[^3H]$-$1,25(OH)_2D_3$ from pig intestinal mucosa expressed VDR.

[2]Relative percent affinity to hDBP ($1,25(OH)_2D_3$ at 100%; 220 nM) determined by competition binding versus $[^3H]$-$1,25(OH)_2D_3$.

[3]Relative percent activity of HL60 differentiation measured by nitroblue tetrazolium assay with 100% (29 nM) indicating the EC50 of $1,25(OH)_2D_3$.

[4]Relative percent activity of MCF-7 growth inhibition determined by $[^3H]$thymidine incorporation with 100% (37 nM) indicating the EC50 of $1,25(OH)_2D_3$.

[5]Relative percent activity of keratinocyte growth inhibition determined by $[^3H]$thymidine incorporation with 100% (5.3 nM) indicating the EC50 of $1,25(OH)_2D_3$.

[6]Relative percent of serum calcium levels in mice after compound treatment with 100% (14.7 mg/dl) indicating $1,25(OH)_2D_3$ at 0.4 µg/kg/day for 7 days.

antiproliferative activity, in that these compounds were able to induce a receptor conformation that was more resistant to protease digestion than $1,25(OH)_2D_3$. These findings support the notion that synthetic nonsteroidal analogs or mimics of the endogenous receptor ligands could induce novel and distinct receptor conformations and that these in turn would lead to altered biological properties.

V. PERSPECTIVES

The application of classic secosteroid VDR ligands to the treatment of many diseases including cancer and osteoporosis has been hampered by the hypercalcemic activity that is observed at the effective pharmacologic doses of these compounds. The identification and characterization of nonsecosteroidal vitamin D mimics indicates that the development of synthetic molecules can lead to differentially regulated VDR activity and

suggests a promising approach to the identification of vitamin D functional analogs with an improved therapeutic index. Surprisingly, among the hundreds of vitamin D analogs that have been generated and reported, there have been very few reports of nonsecosteroid vitamin D ligands. Indeed, there is only one reported structural class, the bisphenol, that is not based on the core $1,25(OH)_2D_3$ template. Recently, another group of nonsecosteroid vitamin D mimics has been disclosed in patent applications (WO 0138320, WO 0138303, WO 02094754), but were not reviewed here due to a lack of available published *in vitro* and *in vivo* data. Other nonsteroidal molecules have been generated through gross structural changes to the $1,25(OH)_2D_3$ molecule in which the C- and D-rings have been eliminated and/or replaced with a novel 5 member E-ring. Nonetheless, based on a molecular and physiological understanding of vitamin D action, these nonsecosteroid compounds display potency and efficacy comparable to $1,25(OH)_2D_3$ with reduced calcemic activity.

TABLE IV   Structures and Activities of Selected E-ring Modifications

Compound	VDR binding[1] (%)	DBP binding[2] (%)	HL60[3] (%)	MCF-7[4] (%)	Kerat[5] (%)	Serum Ca+2;6 (%)
$1,25(OH)_2D_3$	100	100	100	100	100	100
KS 176	10	19	20	30	10	< 0.1
CD 503	80	40	100	850	900	3
CD 504	65	9	80	550	500	2
CD 483	10	3	25	215	400	0.2

[1]Relative percent VDR binding affinity compared to $1,25(OH)_2D_3$ (100%; 0.13 nM) was determined as the concentration of compound needed to displace 50% of [^{3}H]-$1,25(OH)_2D_3$ from pig intestinal mucosa expressed VDR.

[2]Relative percent affinity to hDBP ($1,25(OH)_2D_3$ at 100%; 220 nM) determined by competition binding versus [^{3}H]-$1,25(OH)_2D_3$.

[3]Relative percent activity of HL60 differentiation measured by nitroblue tetrazolium assay with 100% (29 nM) indicating the EC50 of $1,25(OH)_2D_3$.

[4]Relative percent activity of MCF-7 growth inhibition determined by [^{3}H]thymidine incorporation with 100% (37 nM) indicating the EC50 of $1,25(OH)_2D_3$.

[5]Relative percent activity of keratinocyte growth inhibition determined by [^{3}H]thymidine incorporation with 100% (5.3 nM) indicating the EC50 of $1,25(OH)_2D_3$.

[6]Relative percent of serum calcium levels in mice after compound treatment with 100% (14.7 mg/dl) indicating $1,25(OH)_2D_3$ at 0.4 µg/kg/day for 7 days.

Reasons for the improved biological profile of nonseco-steroid compounds include greatly reduced binding to DBP, potentially altered metabolic properties in the case of the bisphenols, and the induction of distinct and unique receptor conformations. These properties can result in altered tissue and gene specificity as compared to the endogenous steroid ligands so that nonsecosteroid VDR ligands represent a scaffold upon which novel compounds can be developed which exhibit unique molecular, pharmacological, and pharmacodynamic properties with the potential for an improved therapeutic index with respect to currently available vitamin D analogs.

Acknowledgments

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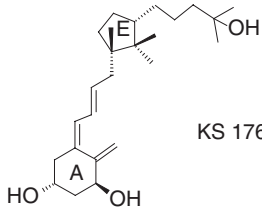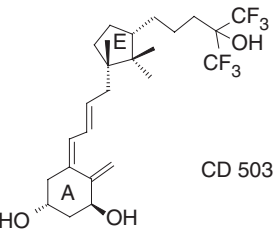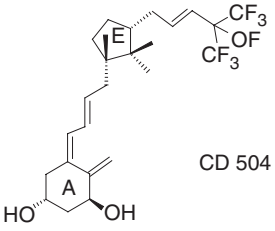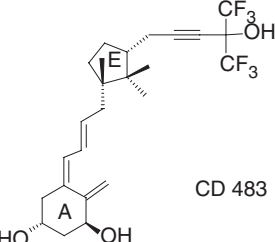4. Stein MS, Wark JD 2003 An update on the therapeutic potential of vitamin D analogs. Expert Opin Investig Drugs **12:**825–840.
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Vitamin D: Cancer and Differentiation

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I. INTRODUCTION

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] is the most potent natural metabolite of vitamin D_3 and is an important regulator of calcium homeostasis and bone metabolism via actions in intestine, bone, kidney, and parathyroid glands. 1,25-(OH)$_2$D$_3$ exerts its effects via an intracellular receptor that is a member of the steroid hormone receptor family (see Chapters 11–20 and 22 in this book). Throughout the last decades, it has become evident that the vitamin D receptor (VDR) is not limited to cells and tissues involved in regulation of calcium and bone metabolism but is also present in a wide variety of other cells and tissues including cancer cells of various origins. This led to a vast series of studies on the role of vitamin D in tumor cell growth regulation, treatment of cancer, and development of potent synthetic vitamin D analogs. Various specialized chapters will discuss in detail the effect of vitamin D on specific cancers (Chapters 89–97) and development and actions of vitamin D analogs (Chapters 80–88). In this chapter we aim to give an overview of the history and current stage and developments on vitamin D and cancer, regulation of tumor cells, possible mechanisms, and clinical applications.

II. VITAMIN D AND CANCER

A. Vitamin D Receptor

As exemplified in Table I, the VDR has also been demonstrated in a broad range of tumors and malignant cell types. For colon and breast cancer cells, an inverse relationship between VDR level and degree of differentiation has been described by some investigators [1,2]. VDR level is increased in ovarian carcinoma compared to normal ovarian tissue [3]. For colorectal cancer it was shown that VDR expression is associated with a more favorable prognosis in colorectal cancer [4]. A VDR immunoreactivity score showed an increase in breast carcinoma specimens compared to normal breast tissue but no clear relation with proliferative status could be assessed [5]. A later study by the same group showed that VDR expression is not a prognostic factor for breast cancer, but the strong VDR immunoreactivity in the breast cancer specimens supports the evidence for it to be a target for intervention [6]. Also in other studies no associations between VDR and clinical and biochemical parameters of breast cancer were found [7–12].

Albeit that the association studies on VDR expression and predictive and/or prognostic characteristics for cancer are so far not conclusive, the widespread distribution of the VDR in malignant cells indicates that regulation of cancer cell function might be a new target in the action of 1,25-(OH)$_2$D$_3$ and provides a biological basis for the epidemiological observations discussed in the next paragraph.

A recent observation put the VDR in relation to cancer in a whole new perspective. It was shown that VDR can function as a receptor for the secondary bile acid lithocholic acid. This compound is hepatotoxic and a potential enteric carcinogenic. Interestingly, both binding of lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [13,14]; (see also Chapter 53). It is postulated that vitamin D and lithocholic acid, by binding to the VDR, activate a feed-forward catabolic pathway that increases CYP3A expression leading to detoxification of carcinogenic bile acids. A relation between the presence of VDR and carcinogenesis was recently also shown for the skin. Absence of VDR increased the sensitivity for chemically induced tumorigenesis [15].

B. Epidemiology

In 1980 an epidemiological study based on indirect evidence suggested a relationship between vitamin D and cancer. This was derived from analyses of death

TABLE I   VDR in Tumors and Malignant Cell Types

Basal cell carcinoma	Myeloid leukemia
Breast carcinoma	Myeloma
Bladder cancer	Osteogenic sarcoma
Cervical carcinoma	Ovarian carcinoma
Colonic adenocarcinoma	Neuroblastoma
Colorectal carcinoma	Non-Hodgkin's lymphoma
Gall bladder carcinoma	Pancreatic carcinoma
Glioma cells	Parathyroid adenoma
Kaposi sarcoma	Pituitary adenoma
Lung carcinoma	Prostate carcinoma
Lymphocytic leukemia	Renal cell carcinoma
Malignant B-cell progenitors	Squamous cell carcinoma
Malignant melanoma	Transitional cell bladder carcinoma
Medullary thyroid carcinoma	Uterine carcinosarcoma

rates from colon cancer, which tended to increase with increasing latitude and decreasing sunlight [16]. Later more direct evidence about a relation between vitamin D and colon cancer came from the inverse relationship between levels of serum 25-hydroxyvitamin D_3 [a $1,25$-$(OH)_2D_3$ precursor] and incidence of colonic cancer [17,18]. In addition, a similar relationship between sunlight exposure, vitamin D, and the risk for fatal breast and prostate cancer has been suggested [19–23] (see Chapter 90). The relationship between sunlight exposure and cancer, especially with respect to vitamin D, has been carefully reviewed by Studzinski and Moore [24]. The dual relationship between sunlight and cancer is of interest and remains the subject of continuing studies [25–27]. A relationship between skin type and prostate cancer has been described [28–30] and recently an article on the skin, sunlight, vitamin D, and cancer has been presented from an evolutionary perspective [31].

The relationship between cancer, diet, and calcium intake and vitamin D has been addressed in several studies [32–37] (see Chapter 91). A Canadian study noted similar vitamin D intakes in breast cancer patients and control subjects [38]. Moreover, in a mouse model, no relationship was found between dietary intake of a wide range of doses of calcium or vitamin D and carcinogen-induced skin tumors [39]. A large Finish epidemiological study showed an association of low serum 25-hydroxyvitamin D_3 with prostate cancer [40,41]. A study on intake of micronutrients suggested that vitamin D and calcium might interact with antioxidants like vitamin C and E in reducing colorectal cancer risk [42]. It is clear that sunlight exposure, vitamin D intake, and other

dietary components such as calcium and fat should be considered as possibly interacting with one another when the relationship between vitamin D and cancer risk is assessed. The data on VDR as bile acid sensor and its postulated role in detoxification provide a direct biological basis for the relation between increased colon cancer and high-fat diets [43] and that colon cancer occurs in areas with higher prevalence of rickets [36]. In addition, mice lacking VDR have been reported to have a higher proliferation rate in the colon [44,45]. A survey of mutations in the VDR in osteosarcomas, several other sarcomas, nonsmall cell lung cancers, and a large number of cell lines representing many tumor types did not show that mutations or rearrangements in the VDR gene play a role in these cancers [46]. Aspects on sunlight and the epidemiology of vitamin D and calcium will be further discussed in greater detail in Chapters 90 and 91, respectively.

In the VDR gene several polymorphisms have been identified and studied in relation to various endpoints (discussed in Chapter 68). Throughout the last years, an increasing number of studies have studied the association of polymorphisms in the VDR and cancer. The first study showed an association between polymorphisms at the 3′ end of the VDR gene and prostate cancer [47]. This was shortly followed by a study showing an association of prostate cancer with variations in the 3′ poly-A stretch in the VDR gene [48]. Interestingly, the Odds Ratio for the VDR polymorphism was about twofold that of the one for the CAG repeat in the androgen receptor. This was followed by several others studies also showing associations of polymorphisms in the 3′ region of the VDR gene and prostate cancer, [49–55] albeit other studies couldn't confirm this [56–60]. For breast cancer both presence [61–66] and absence of association [67] with polymorphisms in the VDR gene have been reported. Also for colon cancer both presence [68,69] and absence [70] of an association with VDR polymorphisms have been reported. No association was reported with basal cell carcinoma [71]. A single study reported an association with the aggressive renal cell carcinoma [72], malignant melanoma [73], and another study on rectal cancer reported a correlation between VDR gene polymorphisms and erbB-2/HER-2 expression [74]. It should be concluded that so far the studies on VDR gene polymorphisms and cancer are far from conclusive. A major reason might be the limited size of most of the studies. More association studies on VDR gene polymorphisms and specific cancers are needed, which should be followed by a meta-analysis to definitively assess whether there is an association and if so, what is the size of the effect. Also, for studies on VDR gene polymorphisms, it is important to take into account the

impact of environmental factors. Diet, vitamin D intake, and sun exposure may modify the association of polymorphism and cancer risk. Interaction between vitamin D and calcium intake and cancer was also found in some of the VDR gene polymorphism studies [68,75–77]. Some studies reported decreased risk of prostate cancer [75] and colorectal adenomas [76] in those subjects with lower vitamin D levels and a particular VDR gene polymorphism. However, results of these studies are unusual in light of the fact that higher calcium and vitamin D intake are generally associated with a modestly reduced risk of colorectal neoplasia. Finally, most importantly it should be realized that except for the FokI translational start site polymorphism, all polymorphisms analyzed so far are anonymous, and functionality or linkage with functional polymorphisms should be proven. The 3′ polymorphisms have been shown to be in linkage with 3′-UTR polymorphisms, but no relation with VDR mRNA stability could be proven [78]. Detailed discussion of possible functional consequences of VDR gene polymorphisms and impact of vitamin D levels is beyond the scope of this chapter but will be addressed in Chapter 67.

C. Growth and Development

In addition to the epidemiological studies and demonstration of vitamin D receptor in tumor cells, since the early 1980s there has also been an increasing amount of cell biological data supporting a role for vitamin D in cancer. Multiple studies have shown that at high concentrations (10^{-9}–10^{-7} M) 1,25-$(OH)_2D_3$ inhibits the growth of tumor cells *in vitro*. It was demonstrated as early as 1981 that 1,25-$(OH)_2D_3$ inhibits the growth of malignant melanoma cells and stimulates the differentiation of immature mouse myeloid leukemia cells in culture [79–81]. 1,25-$(OH)_2D_3$ also induces differentiation of normal bone marrow cells (see Chapter 96). Immature bone marrow cells of the monocyte-macrophage lineage are believed to be the precursors of osteoclasts, and 1,25-$(OH)_2D_3$ induces differentiation of immature myeloid cells toward monocytes-macrophages and also stimulates the activation and fusion of some macrophages (discussed in Chapter 38). From these results, it has been postulated that 1,25-$(OH)_2D_3$ stimulates differentiation and fusion of osteoclast progenitors into osteoclasts [82–84]. Also, in the intestine, 1,25-$(OH)_2D_3$ has important effects on cellular proliferation and differentiation [85]. Thus, via stimulation of the differentiation inducing capacity of bone and interstitial cells, 1,25-$(OH)_2D_3$ may play an important role in the regulation of calcium and bone metabolism. These *in vitro* findings were followed by

the *in vivo* observation that 1,25-$(OH)_2D_3$ prolongs the survival time of mice inoculated with myeloid leukemia cells [86]. As shown in Table II, over the years 1,25-$(OH)_2D_3$ has been shown to have beneficial effects in several other *in vivo* animal models of various types of cancers [87–109].

An important aspect and limitation of the treatment of cancer with 1,25-$(OH)_2D_3$ was revealed by this limited set of clinical trials (see section II.D); to achieve growth inhibition, high doses are needed (confirming the *in vitro* data), which can cause the side effect of hypercalcemia. This has prompted the development of analogs of 1,25-$(OH)_2D_3$ in order to dissociate the antiproliferative effect from the calcemic and bone metabolism effects (see Chapters 80–88) [110,111]. Although the precise mechanism is not completely understood, at the moment several 1,25-$(OH)_2D_3$ analogs are available that seem to fulfill these criteria. In Table III the *in vivo* animal studies using 1,25-$(OH)_2D_3$ analogs on various cancer types are summarized [97,103,104,106–109,112–129].

D. Clinical Studies

Considering the calcemic actions of 1,25-$(OH)_2D_3$ up to this point in time only a few clinical trials of vitamin D compounds in cancer have been performed. Alfacalcidol (1α-hydroxyvitamin D_3; 1α-$(OH)D_3$), which is converted to 1,25-$(OH)_2D_3$ *in vivo,* caused a beneficial response in low-grade non-Hodgkin's lymphoma patients [130,131]. Also, with alfacalcidol, transient improvement in peripheral blood counts was seen in patients with myelodysplasia; however, half of the patients developed hypercalcemia [132]. Another study reported a sustained hematological response in six myelodysplasia patients treated with high doses of alfacalcidol [133]. These patients were restricted in their dietary calcium intake; nevertheless, four patients developed hypercalcemia due to increased bone resorption. With respect to treatment of cutaneous T-cell lymphoma with a combination of 1,25-$(OH)_2D_3$ and retinoids, contrasting results have been obtained. It has been suggested that the variability was due to differences in phenotype of the various lymphomas [134–138]. A study on early recurrent prostate cancer showed that daily treatment with 1,25-$(OH)_2D_3$ slowed the rise in prostate-specific antigen, but treatment coincided with hypercalcemic affects [139]. Using a regime of weekly treatment with high-dose calcitriol was found to be safe, but didn't result in a significant reduction in prostate-specific antigen (PSA) in prostate cancer cells [140]. Two studies were specifically designed to examine the route of application and calcemic response in patients with advanced malignancies [141,142].

TABLE II   *In Vivo* Effects of 1,25-(OH)$_2$D$_3$ and 1α-(OH)D$_3$ in Animal Models of Cancer[a]

Tumor	Model	Effect	Refs.
Adenocarcinoma	CAC-8 cells injected in nude mice	Reduction in tumor volume	[107]
Breast	NMU- and DMBA-induced breast cancer in rats	Tumor suppression	[93,96]
Colon	Human colon cell line implanted into nude mice; DMH-induced colon cancer in rats; APCmin mice	Tumor suppression; reduction of the incidence of colon adenocarcinomas; decrease in polyp number and tumor load	[90,92,95,371]
Kaposi sarcoma	KS Y-1 cells implanted in nude mice	Tumor growth retardation	[105]
Leydig tumor	Leydig cell tumor implanted into rats	Tumor suppression	[97]
Lung	Implantation of lewis lung carcinoma into mice	Reduction of the number of metastases (without suppression of primary tumor); tumor suppression; increased antitumor immunity	[87,99,101,102]
Melanoma	Human melanoma cells implanted into nude mice	Tumor suppression	[90]
Osteosarcoma	Human osteosarcoma cells implanted into nude mice	Tumor suppression	[98]
Prostate	Dunning MAT LyLu rat prostate model; LNCaP xenografts in nude mice; PAIII tumors in Lobund-Wistar rats	Reduction in lung metastasis; tumor suppression	[103,104,106,108,109]
Retinoblastoma	Retinoblastoma cell line implanted into nude mice; transgenic mice with retinoblastoma	Tumor suppression	[91,94]
Walker carcinoma	Walker carcinoma cells injected in rats	Tumor suppression	[100]
Skin	DMBA/TPA-induced skin tumors in mice	Inhibition of tumor formation	[88,89]

[a]The dosage, duration of treatment, diet, and effects on serum/urinary calcium vary among the studies. NMU, Nitrosomethylurea; DMBA, 7,12-dimethylbenz[a]anthracene; DMH, 1,2-dimethylhydrazine dihydrochloride; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Clinical trials using vitamin D analogs have been initiated over the last years. However, these were mostly limited clinical trials focusing on small groups of patients for whom regular treatment has failed. Only data from a few studies has been published. The analog calcipotriol (MC903) has been used for topical treatment of advanced breast cancer; however, several of the patients still developed hypercalcemia [143]. More recent studies have been published on advanced breast cancer [144] and pancreatic cancer [145] but the clinical results were limited. In a single case of Kaposi sarcoma and topical application of calcipotriol (Daivonex/Dovonex/MC903), good success in tumor regression was reported [105]. In Chapter 97 the current clinical status of 1,25-(OH)$_2$D$_3$ and its analogs as therapeutic agents for cancer will be discussed in greater detail.

E. Angiogenesis and Metastasis

For the tumor suppressive activity of vitamin D$_3$ compounds *in vivo*, besides growth inhibition, two additional actions may be involved. First, angiogenesis is an essential requirement for the growth of solid tumors. Compounds that inhibit angiogenesis might therefore contribute to antitumor therapy. Antiangiogenic drugs may cause inhibition of tumor progression, stabilization of tumor growth, tumor regression, and prevention of metastasis. Antiangiogenic effects may play a role in the tumor suppressive activity of vitamin D$_3$ compounds. Two studies reported an antiangiogenic effect of 1,25-(OH)$_2$D$_3$ and the analog 22-oxacalcitriol using different experimental model systems [115,146]. In addition, it was shown that 1,25-(OH)$_2$D$_3$ inhibits angiogenesis induced by the human papilloma virus type 16 (HPV16)- or HPV18-containing cell lines HeLa. Skv-e2, and Skv-el2 when intradermally injected into immunosuppressed mice [147]. Also, with the non-virus-transformed human cell lines T47-D (breast carcinoma) and A431 (vulva carcinoma), similar results were obtained [148]. In these studies the mice were treated for 5 days with 1,25-(OH)$_2$D$_3$ prior to the injection of tumor cells. The effect of 1,25-(OH)$_2$D$_3$ on angiogenesis may be due to inhibition of tumor cell proliferation, resulting in fewer angiogenic cells.

TABLE III *In Vivo* Effects 1,25-(OH)$_2$D$_3$ Analogs in Animal Models for Cancer[a]

Analog	Model	Antitumor effect	Refs.
1,25-(OH)D$_2$	Retinoblastoma	Tumor suppression	[128]
1,25-(OH)D$_5$	Breast	Tumor suppression	[129]
CB966	Breast	Tumor suppression	[114]
CB1093	Prostate	Tumor suppression	[108]
		No effect on angiogenesis	
DD-003	Colon	Tumor suppression	[120]
EB1089	Adenocarcinoma	Tumor suppression	[107]
EB1089	Breast	Tumor suppression	[114,116,125,316]
EB1089	Colon	Tumor suppression	[124]
EB1089	Hepatocellular carcinoma	Inhibition of tumor incidence	[372]
EB1089	Leydig cell tumor	Tumor suppression	[97]
EB1089	Prostate	Tumor suppression	
		Reduction lung metastases	[104,106,108,109,126,127]
		No effect on angiogenesis	
KH1060	Prostate	Tumor suppression	[109]
LG190119	Prostate	Tumor suppression	[106]
OCT	Breast	Tumor suppression	[113,118]
OCT	Breast	Tumor suppression	[115]
OCT	Breast	Tumor suppression	[118]
OCT	Colon	Decreased tumor incidence	[121]
MC903	Breast	Tumor suppression	[117]
Ro 23-7553	Prostate	Tumor suppression	[122]
Ro 23-7553	Leukemia	Increased survival	[112]
Ro 24-5531	Breast	Decreased tumor incidence	[119]
Ro 24-5531	Colon	Decreased tumor incidence	[123]
Ro-25-6760	Prostate	Tumor suppression	[103]
Ro-26-9114	Colon	Decrease in polyp number and tumor load	[371]
Ro-26-9114	Prostate	Tumor suppression	[109]

[a]MC903, 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D$_3$; CB966, 24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D$_3$; CB1093, 20-epi-22(S)-ethoxy-23yne-24a, 26a,27a-trihomo-1α,25-dihydroxyvitamin D$_3$; DD-003,22(S)-24-homo-26,26,26,27,27,27-hexafluoro-1α,22,25-trihydroxyvitamin D$_3$; EB1089, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D$_3$; OCT, 22-Oxacalcitriol; Ro 23-7553, 1,25-dihydroxy-16-ene-23-yne-vitamin D$_3$; Ro 24-5531, 1,25-dihydroxy-16-ene-23-yne-26,27-hexafluorovitamin D$_3$. Ro 26-9114, 1α,25-(OH)$_2$-16-ene-19-nor-24-oxo-D$_3$.

However, inhibition of angiogenesis could also be observed when the tumor cells were treated *in vitro* with 1,25-(OH)$_2$D$_3$ and, after cell washing, were injected into mice [148]. Under these conditions both control and 1,25-(OH)$_2$D$_3$-treated mice were injected with similar numbers of cells. Therefore, these data indicate that 1,25-(OH)$_2$D$_3$ inhibits the release of angiogenic factors (vascular endothelium growth factor, transforming growth factor-α, basic fibroblast growth factor, epidermal growth factor, etc.) or stimulates antiangiogenic factors. 1,25-(OH)$_2$D$_3$ treatment caused a reduction in the angiogenic signaling molecule, angiopoietin-2 in squamous cell carcinoma and radiation-induced fibrosarcoma-1 cells [149]. In retinoblastomas in mice, 1,25-(OH)$_2$D$_3$ has also been shown to reduce angiogenesis [150]. A recent study by Oades *et al.*, however, showed that the 1,25-(OH)$_2$D$_3$ analogs EB1089 and CB1093 inhibited tumor growth in two prostate animal models but did not inhibit angiogenesis in a rat aorta assay [108]. Whether this indicates that vitamin D affects angiogenesis in a tumor situation and not in a nonmalignant condition is not clear. This may resemble the effects of endostatin, which inhibits pathological but not normal

vascularization [151,152]. In support of this is the finding that 1,25-$(OH)_2D_3$ and its analogs EB1089, Ro-25-6760, and ILX23-7553 potently inhibit growth of endothelial cells derived from tumors, but are less potent against normal aortic or yolk sac endothelial cells [149]. Finally, an interesting observation is deglycosylated vitamin D–binding protein (DBP-maf) has also been reported to inhibit angiogenesis [153,154] and to inhibit growth of pancreatic tumor in nude mice [154]. Whether 1,25-$(OH)_2D_3$ may interfere with DBP-maf in tumor growth inhibition and antiangiogenesis remains to be established. Interaction with another factor, interleukin-12, in the inhibition of angiogenesis has been reported [155].

The second mechanism of antitumor activity, which may be related to angiogenesis, is metastasis. Metastasis is the primary cause of the fatal outcome of cancer diseases. A study by Mork Hansen *et al.* indicated that 1,25-$(OH)_2D_3$ may be effective in reducing the invasiveness of breast cancer cells [156]. They showed that 1,25-$(OH)_2D_3$ inhibited the invasion and migration of a metastatic human breast cancer cell line (MDA-MB-231) using the Boyden chamber invasion assay. In support of this, it was shown that 1,25-$(OH)_2D_3$, KH1060, EB1089, and CB1093 inhibited secretion of tissue-type and urokinase plasminogen activator and increased plasminogen activator inhibitor 1 in the MDA-MB-231 metastatic breast cancer cells [157]. In an *in vivo* study, it was shown that 1,25-$(OH)_2D_3$ reduces metastasis to the lung of subcutaneously implanted Lewis lung carcinoma cells [101]. In two animal models of prostate cancer, 1,25-$(OH)_2D_3$ and the analogs EB1089 and RO25-6760 inhibited lung metastases [103,104]. In these models, the tumors were implanted subcutaneously and therefore, in contrast to the model of direct tumor cell injection in the left ventricle [158], no bone metastases occurred. However, a fact to be considered in relation to metastasis is that bone is the most frequent site of metastasis of advanced breast and prostate cancer. There are some indications from clinical studies that bone metastases develop preferentially in areas with high bone turnover [159,160]. In contrast, agents that inhibit bone resorption have been reported to reduce the incidence of skeletal metastasis [161]. As 1,25-$(OH)_2D_3$ may stimulate bone turnover, treatment of cancer with 1,25-$(OH)_2D_3$ might theoretically increase the risk of skeletal metastases. This aspect of 1,25-$(OH)_2D_3$ therapy certainly needs further study. In this aspect, the use of vitamin D_3 analogs with reduced calcemic activity or treatment with vitamin D_3 in combination with other compounds to reduce bone turnover (see Section IV) may be helpful. The data obtained so far on angiogenesis and metastasis indicate that these two processes are part of the spectrum of mechanisms by which vitamin D_3 exerts its anticancer activity.

F. Parathyroid Hormone-Related Peptide

1,25-$(OH)_2D_3$ and parathyroid hormone (PTH) mutually regulate synthesis and secretion of one another. Production and secretion of PTH are inhibited by 1,25-$(OH)_2D_3$ via a transcriptional effect, and a vitamin D responsive element (VDRE) in the promoter of the PTH gene has been identified [162,163] (see Chapter 30). Parathyroid hormone-related peptide (PTHrP) was initially isolated from several carcinomas and is responsible for the humoral hypercalcemia of malignancy syndrome [164]. Although originally identified in carcinomas, PTHrP has also been identified in normal cells (see Chapter 43).

In normal human mammary epithelial cells, 1,25-$(OH)_2D_3$ did not affect basal but inhibited growth factor-stimulated PTHrP expression via an effect on transcription [165]. In normal keratinocytes 1,25-$(OH)_2D_3$ had no effect on PTHrP secretion in basal culture conditions [166], but did inhibit growth factor-stimulated PTHrP production as well [167]. Likewise, 1,25-$(OH)_2D_3$, as well as the analogs 22-oxacalcitriol and MC903, inhibited PTHrP secretion in immortalized human keratinocytes (HPK1A), but this inhibition was less in the more malignant ras-transfected clone HPK1A-ras [168,169]. 1,25-$(OH)_2D_3$ and the analogs EB1089 and 22-oxacalcitriol inhibit the PTHrP gene transcription in and release from the squamous cancer cell line NCI H520 [170]. In addition, in the human T-cell lymphotrophic virus type I (HTLV-I)-transfected T-cell line MT-2, 1,25-$(OH)_2D_3$ and 22-oxacalcitriol did inhibit PTHrP gene expression and PTHrP secretion [171]. In rat H-500 Leydig tumor cells [172], and PC-3 prostate cancer cells 1,25-$(OH)_2D_3$ inhibited PTHrP secretion. It was suggested that this might play a role in the growth inhibition by vitamin D as PTHrP stimulates prostate cancer growth, tumor invasion, and metastasis [173–175]. *In vivo* observations comparable to these *in vitro* observations have also been made. When these H-500 Leydig tumor cells were implanted in Fisher rats, treatment with 1,25-$(OH)_2D_3$ and the analog EB1089 resulted in reduced levels of tumor PTHrP mRNA and PTHrP serum levels [97]. EB1089 also reduced serum levels of PTHrP in nude mice implanted with squamous cancer cells [176]. In Fisher rats implanted with the Walker carcinoma, 1,25-$(OH)_2D_3$ caused a decrease in serum PTHrP, but the ratio of PTHrP levels and tumor weight was similar in rats receiving vehicle or 1,25-$(OH)_2D_3$. The data point to an indirect effect on PTHrP via growth inhibition. However, the PTHrP mRNA levels appeared to be decreased by 1,25-$(OH)_2D_3$ [100]. In nude mice bearing the FA-6 cell line of a pancreas carcinoma lymph node metastasis, 22-oxacalcitriol inhibits PTHrP gene expression, which is related to inhibition of

tumor-induced hypercalcemia [177]. Together, the overall picture that emerges from these studies is that an important additional anticancer effect of vitamin D_3 and analogs could be the inhibition of the humoral hypercalcemia of malignancy.

In contrast to these inhibitory effects in human tumor cells and tumor models, a stimulatory effect of 1,25-$(OH)_2D_3$ and EB1089 on PTHrP gene transcription and PTHrP production by a canine oral squamous carcinoma cell line (Sec 2/88) has been observed [178,179]. Also in an *in vivo* model of canine adenocarcinoma CAC-8 implanted in nude mice, stimulation of PTHrP by 1,25-$(OH)_2D_3$ and EB1089 was observed [179]. These data indicate that the effect of vitamin D and analogs on canine tumors differs from that on human tumors.

III. VITAMIN D EFFECTS ON TUMOR CELLS

A. Cell Cycle

It has now been well established that vitamin D inhibits growth of cells by interfering with the cell cycle. Proliferating cells progress through the cell cycle, which comprises the G_0/G_1 phase (most differentiated, nondividing cells are in the G_1 phase), the S phase in which new DNA is synthesized, and the G_2 phase, which is followed by mitosis (M phase) whereon the cells reenter the G_0/G_1 phase. In most of the cells studied so far, treatment with 1,25-$(OH)_2D_3$ and its analogs results in a blockade at a specific checkpoint, i.e., the restriction point (R), in the G_1 phase limiting the transition of G_1 to S and reducing the number of cells in S phase. Some studies also have examined the effect on the G_2 phase, but these results are somewhat more diverse. In general it can be concluded that blocking the transition from the G_0/G_1 phase to the S phase plays an important role in the growth inhibitory effect of 1,25-$(OH)_2D_3$.

In the regulation of the cell cycle, numerous genes and proteins have been described. It is beyond the scope of this chapter to discuss in detail the regulation of all of the genes/proteins by vitamin D. In Fig. 1, an overview is given of the interacting genes/proteins that are involved in intracellular signaling and regulating the cell cycle. These genes and proteins are part of the cascade of events on which vitamin D exerts its effects. The components shown to be regulated by vitamin D are indicated. Figure 1 is a compilation of data present so far; it is important to realize that probably not all genes/proteins are affected by vitamin D in all tumor cells. However, in this way one gets an overview of the broad range of effects of vitamin D on intracellular signaling pathways involved in regulation of (tumor) cell

growth. More details on the regulation of the cell cycle will be discussed in several other chapters, especially Chapter 92.

Besides its effects on cell cycle regulation, vitamin D has recently been implicated to be involved in control of genomic stability [180]. 1,25-$(OH)_2D_3$ has been reported to inhibit hepatic chromosomal aberrations and DNA strand breaks [181]. This is supported by the finding that 1,25-$(OH)_2D_3$ and EB1089 stimulated the expression of GADD45, which stimulates DNA repair [182] and might be coupled to release of p53 from Mdm2 (see Fig. 1).

1. (ONCO)GENES AND TUMOR SUPPRESSOR GENES

Oncogenes and tumor suppressor genes generally are involved in control of the cell cycle and apoptosis (see Chapter 92). One of the most widely studied oncogenes in relation to vitamin D is *c-myc*. C-Myc suppresses expression of cell cycle/growth arrest genes gas1, p15, p21, p27, and gadd34, -45, and -153 [183]. C-Myc has been postulated to play an early role in the following cascade of events in G_1: cyclins activate cyclin-dependent kinases (CDKs), which in turn can phosphorylate the retinoblastoma tumor suppressor gene product (p110^{RB}), resulting in transition from G_1 to S phase (see Fig. 1). In HL-60 cells, breast cancer cells, and several other cell types, 1,25-$(OH)_2D_3$ has been reported to decrease *c-myc* oncogene expression [184–189]. Analysis of HL-60 sublines showed a relation between reduction of c-*myc* expression and inhibition of proliferation [190]. Similar observations were made for neuroblastoma cells treated with 1,25-$(OH)_2D_3$, EB1089, and KH10560 [191]. We did not observe a 1,25-$(OH)_2D_3$-induced change in *c-myc* expression in MCF-7 and ZR-75.1 breast cancer cells while they were both growth inhibited [192], and a similar observation has been made for the colon-adenocarcinoma CaCo-2 cell line [193].

Nontransformed embryonic fibroblasts are growth inhibited by 1,25-$(OH)_2D_3$, whereas *c-myc* is not changed or is even increased [194,195]. In the MG-63 osteosarcoma cell line, 1,25-$(OH)_2D_3$ has been shown to enhance *c-myc* expression [196], whereas we observed growth inhibition by 1,25-$(OH)_2D_3$ [197]. These data show that regulation of c-myc expression may be part of growth inhibition by vitamin D, but that this is not generally applicable to all cells. 1,25-$(OH)_2D_3$ has also been reported to regulate expression of other oncogenes, like *c-myb, c-fos, c-fms, c-fra1, c-jun, junD, c-Ki-ras, N-ras, c-src* [189,198–203]; however, these data are rather limited. Nevertheless, it is clear that 1,25-$(OH)_2D_3$ has effects on the expression of various oncogenes. The data so far are not conclusive with respect to which genes are crucial in the growth inhibitory action of 1,25-$(OH)_2D_3$. This can be attributed to the fact that these (proto)oncogenes encode for transcription factors,
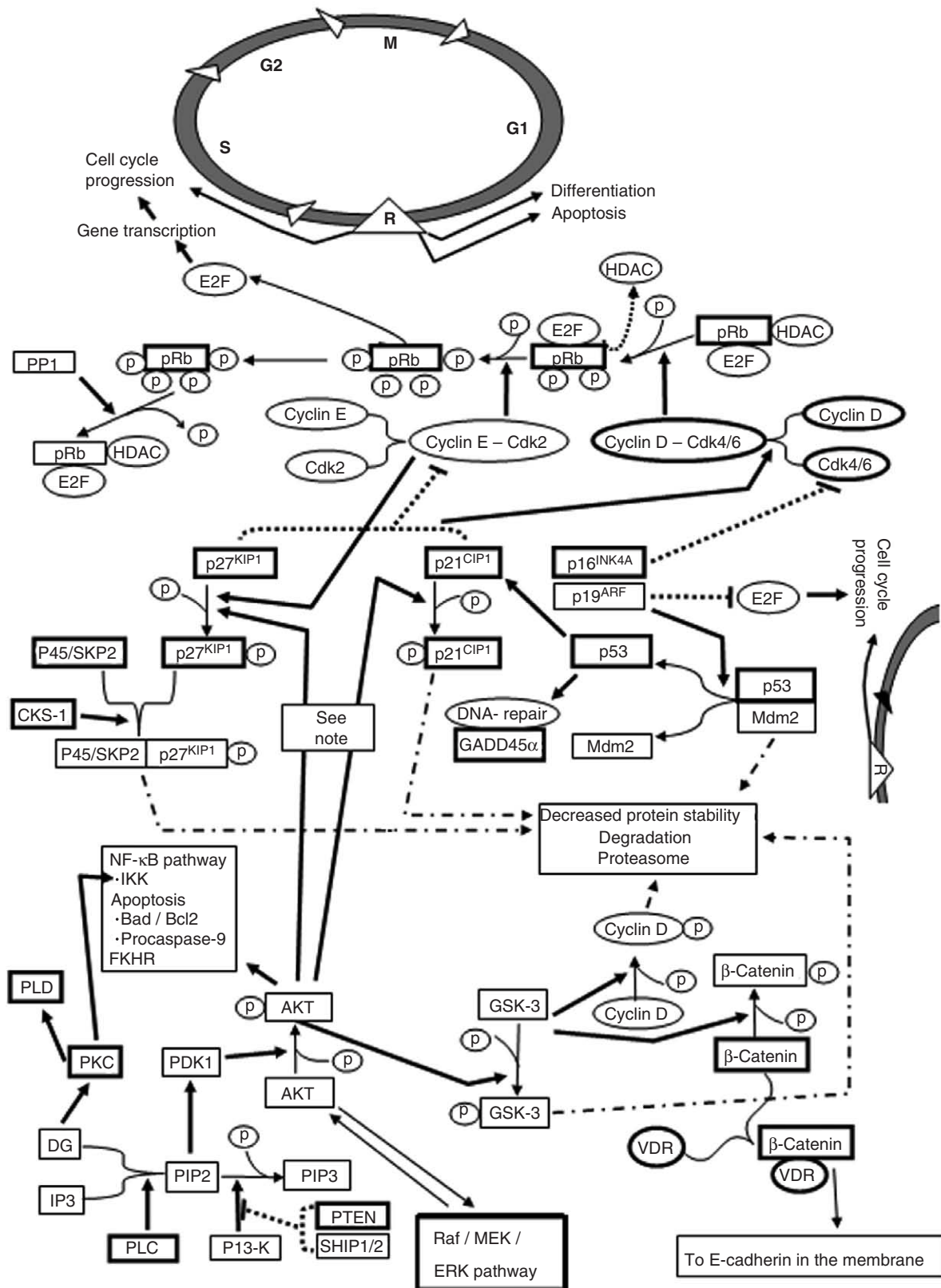
growth factor receptors, or components or intracellular signaling cascades. The effects of these may differ between cells dependent on presence or absence of additional cell type specific conditions. Therefore, their postulated role is often complex. For example, increased *c-myc* expression can be related to induction of apoptosis but also to stimulation of cell cycle progression.

In contrast to the oncogenes, the effect of 1,25-$(OH)_2D_3$ on the retinoblastoma tumor suppressor gene is much clearer. This may be related to the fact that, in contrast to oncogenes, retinoblastoma and p53 take well-defined positions in the control of cell cycle and DNA repair (see Fig. 1). The p110RB retinoblastoma gene product can either be phosphorylated or dephosphorylated. In the phosphorylated form, it can activate several transcription factors and cause transition to S phase and DNA synthesis. In human chronic myelogenous leukemia cells [204], breast cancer cells [205], and HL-60 cells [206,207], 1,25-$(OH)_2D_3$ caused a dephosphorylation of p110RB, which is related to growth inhibition and cell cycle arrest in G_0/G_1 and also in G_2 [207]. In leukemic cells, 1,25-$(OH)_2D_3$ also caused a reduction in the cellular level of p110RB [204,206]. In nontransformed keratinocytes, 1,25-$(OH)_2D_3$ induced dephosphorylation of p110RB as well [208]. The other major tumor suppressor gene is p53. For leukemic U937 cells, it was reported that presence of p53 is important for 1,25-$(OH)_2D_3$-induced differentiation [209]. In rat glioma cells, 1,25-$(OH)_2D_3$ induces expression of p53 [210]. However, 1,25-$(OH)_2D_3$ can inhibit cell growth and induce differentiation in cancer cells with defective p53 [211] and also p53-independent induction of apoptosis by EB1089 has been demonstrated [212]. These latter observations might be explained by the fact that vitamin D also interferes at levels in the cascade of cell cycle control down-stream of p53 (see Fig. 1). Recently, an additional interesting relationship between tumor suppressor genes and vitamin D has recently been shown for the Wilms' tumor suppressor gene WT1. This zinc-finger containing

transcription factor induces transcription of the VDR gene [213].

Several interesting additional genes and vitamin D targets in cancer treatment should be mentioned. First in 1994 Chen and DeLuca isolated and characterized a vitamin D–induced gene in HL-60 cells [214]. This protein, vitamin D-up-regulated protein (VDUP1), is a thioredoxin-binding protein-2 [215]. Thioredoxin has several roles in processes such as proliferation or apoptosis. It also promotes DNA binding of transcription factors such as NF-κB, AP-1, p53, and PEBP2. In addition, overexpression of thioredoxin suppresses the degradation of IκB and the transactivation of NF-κB, whereas overexpression of nuclear-targeted thioredoxin exhibits the enhancement of NF-κB-dependent transactivation [216]. However, it is only in more recent studies that a relationship between VDUP1 and cancer has been established. The expression of VDUP1 was found to correlate with malignant status of colorectal and gastric cancers [217]. 5-fluorouracil, which is widely used for treatment of colon cancer, induces VDUP1 expression in the SW620 colon cancer cell line [218]. In smooth muscle cells and cardiomyocytes VDUP1 inhibits proliferation and is involved in induction of apoptosis [219,220]. An association with vitamin D effects on cancer is made by two recent studies showing induction of VDUP1 by 1,25-$(OH)_2D_3$ in tumor cells and that VDUP1 induces cell cycle arrest [221,222]. Moreover, interaction with histone deacetylase (HDAC; see Fig. 1), promyelocytic leukemia zinc-finger (PLZF) was demonstrated. Interestingly and further complicating the story, PLZF inhibits 1,25-$(OH)_2D_3$ induced differentiation of U937 leukemic cells by binding to the VDR and inhibiting gene transcription [223,224]. Interestingly, the gene, DRH1, was cloned from hepatocellular carcinoma, and its expression was strongly reduced in cancer tissue compared to normal liver [225]. DRH1 has a 41% homology with VDUP1. Whether this points to a new family of cancer genes remains to be established, but it certainly opens new venues for intervening in cancer cell growth.

FIGURE 1   Schematic representation summarizing the intracellular pathways and signaling pathways involved regulation of the cell cycle shown to be regulated by 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs in regulating cell proliferation. Targets shown to be affected by 1,25-$(OH)_2D_3$ and/or its analogs are indicated in the bold boxes and ovals. Bold arrows and fine dotted lines indicate stimulation and inhibition, respectively. Coarse dotted lines indicate processing to the proteasome. p indicates phosphorylation. The effects on these cellular targets are not demonstrated in all types of cancer cells but this diagram is aimed to give an overview of demonstrated targets and potential targets. NOTE: Dependent on the site of phosphorylation proteins can either be destabilized or degraded or be stabilized and activated. For example: phosphorylation of p21 at T145 by AKT leads to degradation while phosphorylation of S146 by AKT leads to increased stability. Abbreviation used: AKT (PKB), Protein kinase B; Bad, BCL2-antagonist of cell death; Bcl2, B-cell leukemia/lymphoma 2; Cdk, Cyclin-dependent kinase; CKS-1, Cyclin kinase subunit 1; DG, Diacylglycerol; E2F, Transcription factor; ERK, Extracellular-signal regulated kinase; FHKR (AFX/FOX), Forkhead family of transcription factors; GSK-3, Glycogen synthase kinase-3; HDAC, Histone deacetylase; IKK, I-κB kinase; IP3, Inositol 1,4,5-trisphosphate; Mdm2, Mouse double minute 2; MEK, Raf-1-MAPK/ERK kinase; PDK1, Phosphatidylinositol-dependent kinase 1; PI3-K, Phosphatidyl inositol 3 kinase; PIP2, Phosphatidylinositol (4,5)-phosphate; PIP3, Phosphatidylinositol (3,4,5) phosphate; PKC, Protein kinase C; PLC, Phospholipase C; PLD, Phospholipase D; PP!, Protein phosphatase 1-like protein; pRB, Retinoblastoma protein; PTEN, Phosphatase and tensin homologue; SHIP 1 and 2, Src homology 2 (SH2) containing phosphatases 1 and 2; SKP2, Ubiquitin ligase; VDR, Vitamin D receptor.

Second, an additional therapeutic target for vitamin D compounds might be regulation of enzymes involved in estrogen and androgen synthesis and metabolism [226–229]. Third, telomerase activity provides a mechanism for unlimited cell division. In HL-60 cells, $1,25\text{-}(OH)_2D_3$ inhibits telomerase activity [230]. Fourth, the homeobox genes may prove to be a major target for vitamin D action in cancer, but this possibility remains to be elucidated. In a differential expression screen using the human U937 leukemic cells, the HoxA10 gene was shown to be regulated by $1,25\text{-}(OH)_2D_3$ [231].

It is to be expected that as a result of the increasing application of large scale microarray gene expression analyses, a vast number of new cell cycle and vitamin D regulated genes will be identified and add to the unraveling and understanding of vitamin D control of cancer cell proliferation [232–235].

B. Apoptosis

A block in the cell cycle preventing transition into S phase may cause cells to go either into apoptosis or to enter a specific differentiation pathway (see Chapter 93). What exactly determines the decision of apoptosis or differentiation remains to be elucidated. It is suggested that early G_1 phase may be the point at which switching between cell cycle progression and induction of apoptosis occurs [236,237].

Induction of apoptosis, an orderly and characteristic sequence of biochemical, molecular, and structural changes resulting in the death of the cell [238], is a mechanism by which $1,25\text{-}(OH)_2D_3$ inhibits tumor cell growth and may contribute to tumor suppression and explain the reduction in tumor volume found in various *in vivo* animal studies (see Section II.C).

$1,25\text{-}(OH)_2D_3$ has been shown to regulate expression of apoptosis genes and to induce apoptosis of cancer cells of various origins. For example, $1,25\text{-}(OH)_2D_3$ and the analog Ro 25-6760 cause a cell cycle block in HT-29 human colon cancer cells, resulting in growth inhibition and induction of apoptosis [239]. The *bcl-2* oncogene decreases the rate of programmed cell death [240,241]. However, protection of HL-60 cells against apoptosis occurred despite down-regulation of *bcl-2* gene expression [242]. In several breast cancer cell lines (MCF-7, BT-474, MDA-MB-231) $1,25\text{-}(OH)_2D_3$ and the analogs KH1060 and EB1089 decreased *bcl-2* expression [211,243]. The analog CB1093 reduced *bcl-2* expression in MCF-7 cells associated with the induction of apoptosis [244]. However, only in MCF-7 cells has this change in *bcl-2* expression been accompanied by apoptosis. Effects on other genes/proteins have also been reported [245], and microarray gene

expression analyses and differential screening will also definitively reveal additional vitamin D targets in regulating apoptosis [246].

A central role for apoptosis in the action of $1,25\text{-}(OH)_2D_3$ is unclear because growth inhibition of several other breast cancer cells appeared to be independent of apoptosis [211]. Also, MCF-7 cells that showed growth inhibition by $1,25\text{-}(OH)_2D_3$ could, after removal of the hormone, again be stimulated to grow, implying transient growth inhibition and not cell death [247]. Stable transfection of leukemic U937 cells with the wild-type p53 tumor suppressor gene resulted in a reduced growth rate and produced cells that can undergo either apoptosis or maturation. In these cells $1,25\text{-}(OH)_2D_3$ protects against p53-induced apoptosis and enhances p53-induced maturation [209]. In two independent studies with HL-60 cells, $1,25\text{-}(OH)_2D_3$ was found either to protect against or to have no effects on apoptosis [242,248]. Vitamin D protection against apoptosis was also detected in human U937 leukemic cells treated tumor necrosis factor α [249]. Absence of a vitamin D effect on apoptosis might be explained by the expression of the antiapoptotic protein BAG-1 p50 isoform. This protein has been shown to bind to the VDR and block vitamin D–induced transcription [250]. The presence of additional interacting factors might also be important for the eventual effect on apoptosis as in the study with HL-60 cells, which in the presence but not the absence of 9-cis-retinoic acid, $1,25\text{-}(OH)_2D_3$ did induce apoptosis [248]. The role of vitamin D interaction with other factors will be discussed in more detail in Section IV. In summary, the data obtained so far show that $1,25\text{-}(OH)_2D_3$-induced growth inhibition can be related to apoptosis in some cases, but that growth inhibition is frequently observed to be independent of apoptosis. Possibly in these latter cases, induction of differentiation is more prominent. The factor that decides whether cells undergo apoptosis or differentiation is unclear but is probably dependent on cell cycle stage, presence of other factors, and levels of expression of oncogenes and tumor suppressor genes. An interesting phenomenom to be studied concerning vitamin D and apoptosis is calbindin 28K. Calbindin 28K is a well-known vitamin D–induced protein that has recently been shown to inhibit apoptosis [251]. It is tempting to speculate that calbindin 28K plays a role in the decision whether vitamin D induces cells to differentiate or to go into apoptosis or that it is involved when $1,25\text{-}(OH)_2D_3$ protects against apoptosis (see Chapter 42).

C. Differentiation

In addition to proliferation and apoptosis, the third major cellular process is differentiation. As described

above for the classic actions of 1,25-$(OH)_2D_3$ related to calcium homeostasis, effects on cell differentiation and proliferation are involved. The coupling between proliferation and differentiation has been most widely studied for cells of the hematopoietic system (Chapter 96) and keratinocytes (Chapter 35). In general, 1,25-$(OH)_2D_3$ inhibits proliferation and induces differentiation along the monocyte-macrophage lineage. Rapidly proliferating and poorly differentiated keratinocytes can be induced to differentiate by 1,25-$(OH)_2D_3$. A further relationship between the vitamin D_3 system and differentiation is demonstrated by the fact that in poorly differentiated keratinocytes 1,25-$(OH)_2D_3$ production and vitamin D receptor levels are high, whereas after induction of differentiation these levels decrease [252], and in melanoma cells 1,25-$(OH)_2D_3$ stimulates melanin production [253]. Effects on differentiation have also been reported for other cell types. Inhibition of prostate cancer cell proliferation is paralleled by an increased production of prostate specific antigen [254–257]. In the BT-20 breast cancer cells 1,25-$(OH)_2D_3$ induced morphological changes indicative for differentiation [258]. In several breast cancer cell lines, the stimulation of differentiation has been established by determining lipid production by the cells [211]. In this study, Elstner *et al.* demonstrated an uncoupling between effects on proliferation and differentiation. In two breast cancer cell lines, 1,25-$(OH)_2D_3$ and various analogs induced differentiation even though the cells were resistant to cell cycle and antiproliferative effects. This finding, together with data obtained with human myelogenous leukemia cells, [204] suggests a dissociation between the cellular vitamin D_3 pathways involved in regulation of differentiation and proliferation (see also Section V). For a HL-60 subclone, a similar observation was made [190], and in another HL-60 subclone the induction of differentiation was found to precede the G_0/G_1 cell cycle block. In contrast to the above-mentioned observations on stimulation of differentiation, 1,25-$(OH)_2D_3$ inhibits erythroid differentiation of the erythroleukemia cell line K562 [186], and 1,25-$(OH)_2D_3$ inhibits Activin A-induced differentiation of murine erythroleukemic F5-5 cells [259]. Although precise relationships among growth inhibition, cell cycle effects, and apoptosis are unclear, it can be concluded that an important effect of vitamin D_3 on both normal and malignant cells is induction of differentiation.

D. Growth Factors and Growth Factor Receptors

Besides regulation of cell cycle-related oncogenes and tumor suppressor genes, interaction with tumor- or stroma-derived growth factors is important for growth inhibition. Stimulation of breast cancer cell proliferation by coculture with fibroblasts is inhibited by 1,25-$(OH)_2D_3$ [260]. A good candidate to interact with the 1,25-$(OH)_2D_3$ action is transforming growth factor-β (TGFβ). TGFβ is involved in cell cycle control and apoptosis [261,262]. TGFβ can interfere with the cascade of events in the GI phase described above and inhibit the ability of cells to enter S phase when the factor is present during the GI phase. TGFβ has been shown to suppress *c-myc,* cyclin A, cyclin E, and cdk2 and cdk4 expression [262]. In line with this, TGFβ has been reported to inhibit phosphorylation of p110^{RB} [263]. Vitamin D_3 compounds induce dephosphorylation of the retinoblastoma gene product, and vitamin D_3 growth inhibition of MCF-7 breast cancer cells is inhibited by a TGFβ neutralizing antibody [264]. 1,25-$(OH)_2D_3$ and several analogs stimulated the expression of TGFβ mRNA and secretion of active and latent TGFβ$_1$ by the breast cancer cell line BT-20 [154]. 1,25-$(OH)_2D_3$ enhanced TGβ$_1$ gene expression in human keratinocytes [265] and the secretion of TGFβ in murine keratinocytes [266]. In both studies, antibodies against TGFβ inhibited the growth inhibitory effect of vitamin D_3. Further evidence for a vitamin D_3-TGFβ interaction is that bone matrix of vitamin D–deficient rats contains substantially less TGFβ than controls [267]. Therefore, on the basis of these consistent findings, TGFβ is a likely candidate to play a role in the l,25-$(OH)_2D_3$-induced growth inhibition [268].

Interactions with the insulin-like growth factor (IGF) system have also been described. IGFs are potent growth stimulators of various cells, and their effect is regulated via a series of IGF binding proteins (IGFBPs). 1,25-$(OH)_2D_3$ and the analog EB1089 inhibit the IGF-I-stimulated growth of MCF-7 breast cancer cells [269]. In prostate cancer cell lines, 1,25-$(OH)_2D_3$ induced expression of IGFBP6 but not IGFBP4 [270]. In human osteosarcoma cell lines, 1,25-$(OH)_2D_3$ and the analog 1α-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol potently stimulated the expression and secretion of IGFBP3 [271–273]. In one study an association has been made between increased IGFBP3 levels and 1,25-$(OH)_2D_3$ growth inhibition [271]. Recent observations that antisense oligonucleotides to IGFBP3 prevented growth inhibition of prostate cancer cells by 1,25-$(OH)_2D_3$ [235] provided further evidence for an interplay between 1,25-$(OH)_2D_3$ and IGFBP3. Interestingly, in the human osteosarcoma cell line MG-63, 1,25-$(OH)_2D_3$ and TGFβ synergistically increased IGF-BP-3 secretion [273]. An example of growth factor receptor regulation by 1,25-$(OH)_2D_3$ concerns the epidermal growth factor (EGF) receptor. This receptor is down-regulated in T47-D breast cancer cells and up-regulated in BT-20 breast cancer cells. Nevertheless, 1,25-$(OH)_2D_3$ inhibits the growth of both

cell lines [274,275]. These data provide evidence that interactions with growth factors are only part of the $1,25\text{-}(OH)_2D_3$ action on tumor cells.

As described above, it is clear that $1,25\text{-}(OH)_2D_3$ has effects on the expression of various oncogenes and tumor suppressor genes and that multiple interactions with various growth factors exist. However, the data on these aspects, separately as well as in combination, are still too limited to define a distinct mechanism of action for the $1,25\text{-}(OH)_2D_3$ anticancer effects. However, with respect to growth inhibition, at this time two models of action can be postulated. In the first one, $1,25\text{-}(OH)_2D_3$ directly interferes with a crucial gene(s) involved in the control of the cell cycle. In this case, in view of the general pattern of the genes involved in cell cycle control, this mechanism of action will be similar in all types of cancer cells. However, the effect on cell cycle genes will be dependent on the presence or absence of additional growth factors. This will determine, depending on which growth factors are present, the differences in $1,25\text{-}(OH)_2D_3$ action between cancer types of different origin but also within cancer types of similar origin. The second model is based on an indirect effect of $1,25\text{-}(OH)_2D_3$ on cell cycle progression and tumor growth. In this case $1,25\text{-}(OH)_2D_3$ may either inhibit or potentiate the effect of growth stimulatory or inhibitory factors, respectively, via, for example, effects on growth factor production, growth factor binding protein levels, or receptor regulation. It is also conceivable that a combination of both models forms the basis of $1,25\text{-}(OH)_2D_3$ regulation of tumor cell growth.

IV. COMBINATION THERAPY

The data obtained with $1,25\text{-}(OH)_2D_3$ and its analogs on growth inhibition and stimulation of differentiation offer promise for their use as an endocrine anticancer treatment. Single agent treatment with low calcemic $1,25\text{-}(OH)_2D_3$ analogs could be useful; however, combination therapy with other tumor effective drugs may provide an even more beneficial effect. Up to now several *in vitro* and *in vivo* studies have focused on possible future combination therapies with $1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ analogs.

For breast cancer cells the combination of the presently most widely-used endocrine therapy, the antiestrogen tamoxifen, with $1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ analogs resulted in a greater growth inhibition of MCF-7 and ZR-75-1 cells than treatment with either compound alone [118,192,247]. In combination with tamoxifen, the cells were more sensitive to the antiproliferative action of $1,25(OH)_2D_3$ and the analogs;

that is, the EC_{50} values of the vitamin D_3 compounds in the presence of tamoxifen were lower than those in the absence of tamoxifen. Studies with MCF-7 cells suggested a synergistic effect of $1,25(OH)_2D_3$ and tamoxifen on apoptosis [276]. In addition, in *in vivo* breast cancer models a synergistic effect of the tamoxifen-$1,25(OH)_2D_3$ analogs combination was observed [118,119]. Additional data on the interaction between the estrogen/antiestrogen system and vitamin D comes from studies showing the presence of an estrogen responsive element in the VDR promoter and regulation of VDR by estradiol in breast cancer cells [277]. This is intriguing that the stimulator of breast cancer cell growth induces the expression of the receptor for a growth inhibitor. VDR up-regulation in breast cancer cells and increased transcriptional activity was mimicked by the phytoestrogens resveratrol and genistein and blocked by tamoxifen [278]. In colon cancer also, VDR up-regulation by estradiol has been reported. However, in colon it was hypothesized to contribute to the protective effect of estradiol on chemically-induced colon carcinogenesis [279].

These important and complex interactions between the vitamin D and estrogen endocrine system in the regulation of cancer (cells) are promising and warrant further detailed analyses, e.g. regarding tissue(cancer)-specific effects. In addition, the estrogen endocrine system may regulate the metabolism of $1,25\text{-}(OH)_2D_3$ in cancer cells and thereby affect its action (see Section V). Interaction with another sex steroid, testosterone, has been described for ovarian cancer. Vitamin D inhibits dihydrotestosterone (DHT) and DHT stimulation of ovarian cancer cells [280]. Intriguingly, also here the growth stimulator and growth inhibitor mutually up-regulate each others receptors. Also, in prostate cancer cells, it has been shown that $1,25\text{-}(OH)_2D_3$, while inhibiting androgen stimulated growth, up-regulates the androgen receptor [281].

Interaction with another steroid in regulating cancer cells had already been reported in 1983. The synthetic glucocorticoid, dexamethasone, and $1,25\text{-}(OH)_2D_3$ synergistically induced differentiation of murine myeloid leukemia cells [282]. This was supported by *in vitro* and *in vivo* data showing that dexamethasone enhanced the effect of vitamin D on growth inhibition, cell cycle arrest, and apoptosis of squamous carcinoma cells [283,284]. A possible mechanism is the up-regulation of VDR by dexamethasone [283]. An interesting aspect of this combination is not only the direct interaction at cancer cell level, but also in the control of the calcemic action of $1,25\text{-}(OH)_2D_3$. Glucocorticoids inhibit intestinal calcium absorption and increase renal calcium excretion and in this way it may limit the hypercalcemic action of $1,25\text{-}(OH)_2D_3$ [285].

Combination of vitamin D_3 and retinoids has been examined in various systems. A combination of retinoic acid and 1,25-$(OH)_2D_3$ resulted in a more profound inhibition of both T47-D breast cancer cells [286] and LA-N-5 human neuroblastoma cells [287]. 9-cis-Retinoic acid augmented l,25-$(OH)_2D_3$-induced growth inhibition and differentiation of HL-60 cells [288]. Besides growth inhibition and differentiation effects, the combination of 1,25-$(OH)_2D_3$ and various isomers of retinoic acid were more potent in reducing angiogenesis than either compound alone [146–148]. The background of the interaction between retinoids and 1,25-$(OH)_2D_3$ may be attributed to heterodimer formation of the respective receptors [289].

For several cytokines, interactions with 1,25-$(OH)_2D_3$ have been described. Interferon-γ and 1,25-$(OH)_2D_3$ synergistically inhibited the proliferation and stimulated the differentiation of HL-60, WEHI-3, and U937 myeloid leukemia cells [290–293]. Treatment of LLC-LN7 tumor cells with 1,25-$(OH)_2D_3$ with IFN-γ synergistically reduced tumor granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion and a blockage in the capacity of the tumor cells to induce granulocyte-macrophage-suppressor cells [99]. In the mouse myeloid leukemia cell line Ml interleukin-4 enhanced 1,25$(OH)_2$ D_3-induced differentiation [189,294,295]. Also with interleukin-1β, interleukin-3, interleukin-6, and interleukin-12 interactions with 1,25-$(OH)_2D_3$ have been reported [296–298]. 1,25-$(OH)_2D_3$ and tumor necrosis factor synergistically induced growth inhibition and differentiation of HL-60 [299]. For MCF-7 cells an interaction between 1,25-$(OH)_2D_3$ and tumor necrosis factor has also been reported [298,300]. In the presence of GM-CSF, lower concentrations of 1,25$(OH)_2D_3$ could be used to achieve a similar antiproliferative effect in MCF-7 cells [301] and to induce differentiation of U937 myeloid leukemic cells [302]. Other factors shown to interact with 1,25-$(OH)_2D_3$ are butyrate [303–305], melatonin [306], EGF [307], and the factors described in Section III.C.

Furthermore, combinations of vitamin D_3 compounds with cytotoxic drugs, antioxidants, and radiation have been studied. *In vivo* adriamycin and *in vitro* carboplatin, cisplatin, and doxorubicin interacted synergistically with 1,25-$(OH)_2D_3$ to inhibit breast cancer cell growth [113,308–311]. In a carcinogen-induced rat mammary tumor model, treatment with 1α-$(OH)D_3$ and 5-fluorouracil, however, did not result in enhanced antitumor effects [96]. Recently, interactions with a plant-derived polyphenolic antioxidant, carnosic acid were demonstrated in the differentiation of HL-60 cells, which was related to a decrease in the intracellular levels of reactive oxygen species [312,313]. Also interaction with radiation therapy in breast cancer has been described [314–316].

The data on combinations of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs with various other anticancer compounds are promising and merit further analyses. The development of effective combination therapies may result in better response rates and lower required dosages, thereby reducing the risk of negative side effects.

V. RESISTANCE AND VITAMIN D METABOLISM

Classic vitamin D resistance concerns the disease hereditary vitamin D–resistant rickets, which is characterized by the presence of a nonfunctional VDR and consequently aberrations in calcium and bone metabolism (see Chapter 72). For cancer cells, the presence of a functional VDR is also a prerequisite for a growth regulatory response, and a relationship between VDR level and growth inhibition has been suggested for osteosarcoma, colon carcinoma, breast cancer, prostate cancer cells, and rat glioma [1,2,108,129,205,210,317–321]. Cell lines established from DMBA-induced breast tumors in VDR knockout mice are insensitive to growth arrest and apoptosis by 1,25-$(OH)_2D_3$, EB1089 and CB1093 [322]. Albeit that VDR is a prerequisite for tumor cell growth regulation, the presence of the VDR is not always coupled to a growth inhibitory response of 1,25$(OH)_2D_3$. Results from studies with transformed fibroblasts [194], myelogenous leukemia cells [190,204,323], transformed keratinocytes [187], and various breast cancer cell lines [211,324] demonstrated a lack of growth inhibition by 1,25$(OH)_2D_3$ even in the presence of VDR. In this situation, the designation "resistant" is based on the lack of growth inhibition, even though, as discussed earlier in Section III.C, some of these cells are still capable of being induced to differentiate [204,211]. This points to a specific defect in the growth inhibitory pathway. In the resistant MCF-7 cells, this defect is not located at a very common site in the growth inhibitory pathway of the cell because the growth could still be inhibited with the antiestrogen tamoxifen [324]. For myelogenous leukemia cells, similar observations have been made [325].

For VDR-independent resistance to growth inhibition, the underlying mechanism(s) is unknown. For the resistant MCF-7 clone, this is not related to up-regulation of the P-glycoprotein [324]. Interestingly, these vitamin D–resistant MCF-7 clones can be sensitized to 1,25$(OH)_2D$ by activation of protein kinase C, resulting in induction of apoptosis and transcriptional activation, suggesting that alterations in phosphorylation may affect vitamin D sensitivity [326]. An interesting growth

inhibition resistant MCF-7 cell clone was described by Hansen *et al*. This clone was not growth inhibited while VDR was still present and 24-hydroxylase could still be induced [327]. Other examples of vitamin D resistance are HL60 cells that have been cultured for four years in the presence of 1,25-$(OH)_2D_3$ and resulted in clones that are resistant to differentiation inducing and growth inhibition. They became not only resistant to 1,25$(OH)_2D$ but also to 5-beta-D-arabinocytosine, suggesting a common metabolic pathway being responsible [328]. Whether this relates to the up-regulation of the multidrug resistance proteins is not clear. In the resistant leukemia $JMRD_3$ cell line, altered regulation and DNA–binding activity of *junD* as part of the AP-1 complex has been reported [200]. Resistance to growth inhibition in the presence of VDR has also been linked to disruption of the VDR-RXR complex [329] and increased RXR degradation [330]. In addition, other factors, like the acute myeloid leukemia translocation products (e.g. PLZF) may contribute to resistance to vitamin D by sequestering the VDR [223,224].

The 1,25$(OH)_2D_3$ sensitive and resistant cell clones provide interesting models to examine the molecular mechanisms of l,25$(OH)_2D_3$-induced growth inhibition. For example, lack of p21 results in no cell cycle block [331] and no apoptosis was detected with a mutated p53 [211]. Finally, the recent identification of cellular proteins that are involved in the vitamin D resistance in new world primates might add to the understanding of tumor cell resistance to vitamin D [332,333] (see Chapter 21).

At this time, the major mechanism for vitamin D resistance or reduced sensitivity in VDR containing tumor and cancer cells is 1,25-$(OH)_2D_3$ catabolism via the C24-hydroxylation pathway. An inverse relationship between cellular metabolism of 1,25-$(OH)_2D_3$ via 24-hydroxylation and growth inhibition of prostate cancer cells has been suggested [318]. The latter observation is intriguing, the more so as an inverse relationship between VDR level and induction of 24-hydroxylase (CYP24) activity was reported. In general, there may exist a direct relationship between VDR level and induction of 24-hydroxylase activity [319,334]. An important role in the control of 1,25-$(OH)_2D_3$ action on cancer cells was provided by studies with the 1,25-$(OH)_2D_3$–resistant prostate cancer cell line DU145. It was shown that 1,25-$(OH)_2D_3$ did inhibit the growth of these cells when it was combined with the 24-hydroxylase inhibitor Liazorole [335]. Inhibition of 24-hydroxylase activity in HL-60 cells also altered the effect of 1,25-$(OH)_2D_3$ and 20-epi analogs [336]. The action of the analog EB1089 was also limited by hydroxylation at the C24 position [337]. However, it was

suggested that the increased potency of EB1089 is at least partly due to resistance to 24-hydroxylation [234]. Alternatively, 24-hydroxylation of the analog KH1060 has been implicated as one of the mechanisms to explain the potency of this analog. The 24-hydroxylated metabolites of this analog are very stable and are biologically active [338,339]. It has been shown that the naturally occurring 24-hydroxylated metabolite of vitamin D_3, 24R,25-$(OH)_2D_3$, also has a preventive effect on chemically-induced colon cancer [340].

Interaction between the estrogen system and 24-hydroxylase is also of importance. Recent data have shown that the phytoestrogen genistein inhibits 24-hydroxylase activity in prostate cancer cells and thereby increases the responsiveness to 1,25-$(OH)_2D_3$ [341]. A role for 24-hydroxylase as oncogene is suggested by data showing amplification of the CYP24 locus on chromosome 20q13.2 [342].

In contrast to degradation of 1,25-$(OH)_2D_3$ by 24-hydroxylase in cancer cells, recently it has become clear that tumor cells contain 1α-hydroxylase activity and thereby are able to generate 1,25-$(OH)_2D_3$. Expression of 1α-hydroxylase has been demonstrated in colorectal cancer [343–345]. It was postulated that in early stages tumor cells respond by up-regulating 1α-hydroxylase activity to counteract neoplastic growth while at later stages of tumor development this is lost [343]. Also in prostate cancer [346] and inflammatory myofibroblastic tumor [347] 1α-hydroxylase has been detected, albeit in the latter case the tumor contains large numbers of macrophages. It can be anticipated that in the coming years investigation of the expression of both 24-hydroxylase, 1α-hydroxylase in tumors will add to the understanding of vitamin D in the initiation and progression of cancer.

VI. STIMULATION OF PROLIFERATION

Over the years a limited number of studies have demonstrated that, in contrast to growth inhibition, 1,25-$(OH)_2D_3$ can also stimulate tumor cell growth and tumor development. In several cells 1,25-$(OH)_2D_3$ has been reported to have a biphasic effect, that is, at lower concentrations ($<10^{-9}$ M) it stimulates proliferation and at higher concentrations (10^{-9} to 10^{-7} M) it inhibits proliferation. However, clear growth stimulation can sometimes be observed not only at low concentrations but also at the concentrations generally found to inhibit tumor cell proliferation and tumor development. 1,25-$(OH)_2D_3$ has been shown to stimulate the growth of a human medullary thyroid carcinoma cell line [348]. Not only cancer cells but also several normal cells, for example, human monocytes [349], smooth muscle

cells [350], and alveolar type II cells [351], are stimulated to grow by 1,25-(OH)$_2$D$_3$.

Skin is another organ in which different effects of 1,25-(OH)$_2$D$_3$ have been observed. *In vivo* studies demonstrated that 1,25-(OH)$_2$D$_3$ and analogs stimulate keratinocyte proliferation in normal mice [352–355] and enhance anchorage-independent growth of preneoplastic epidermal cells [356]. In contrast, other studies showed 1,25-(OH)$_2$D$_3$ inhibition of proliferation of mouse and human keratinocytes [357,358], and 1,25-(OH)$_2$D$_3$ is also effective in the treatment of the hyperproliferative disorder psoriasis [359]. Moreover, *in vivo* studies demonstrated that, depending on the carcinogen, 1,25-(OH)$_2$D$_3$ can either reduce [88] or enhance the induction and development of skin tumors in mice [360,361]. In addition, 1,25-(OH)$_2$D$_3$ enhances the chemically-induced transformation of BALB 3T3 cells and hamster embryo cells [362,363]. 1,25-(OH)$_2$D$_3$ also enhanced 12-O-tetradecanoylphorbol-13-acetate-induced tumorigenic transformation of mouse epidermal JB6 Cl41.5a cells [364,365].

Another example comes from research on osteosarcoma cells. In 1986 it was shown that 1,25-(OH)$_2$D$_3$ stimulated the growth of tumors in athymic mice inoculated with the ROS 17/2.8 osteosarcoma cell line [366]. Earlier the same group reported growth stimulation *in vitro* of these osteosarcoma cells at low concentrations of 1,25(OH)$_2$D$_3$, but growth inhibition by 10^{-8} M [317]. They speculated that this discrepancy resulted from limited *in vivo* availability of 1,25-(OH)$_2$D$_3$ for the tumor cells, resulting in concentrations shown to be growth stimulatory *in vitro*. However, in other experiments with nude mice, the availability of 1,25-(OH)$_2$D$_3$ did not seem to be a factor, as growth inhibition was observed

(see Table II). In particular, in nude mice implanted with human osteosarcoma cells (MG-63), growth inhibition and tumor suppression by 1,25-(OH)$_2$D$_3$ were observed [98]. In two different *in vitro* studies, growth inhibition of MG-63 and growth stimulation of ROS 17/2.8 cells was reported [367,368]. For smooth muscle cells, it has been demonstrated, for example, that growth inhibition or stimulation can depend on the presence of additional growth factors in the culture medium [350]. We followed up on this concept by comparing the effects of 1,25-(OH)$_2$D$_3$ and analogs on the growth and osteoblastic characteristics of the two osteosarcoma cell lines under identical culture conditions. At concentrations 10^{-10} to 10^{-7} *M,* 1,25-(OH)$_2$D$_3$ caused an increase in cell proliferation by 100% in ROS 17/2.8 cells, whereas the proliferation of MG-63 cells was inhibited (Fig. 2) [197]. In contrast, in both cell lines 1,25(OH)$_2$D$_3$ stimulated osteoblastic differentiation characteristics such as production of osteocalcin and alkaline phosphatase activity [197,367]. Analyses with another steroid hormone demonstrated that glucocorticoids inhibited the growth of both osteosarcoma cell lines [369,370]. These data indicate specific differences between these cell lines, especially with respect to the 1,25-(OH)$_2$D$_3$ growth regulatory mechanisms.

Taken together, the data on growth stimulation and tumor development, although detected in only a minority of cancer cells, demonstrate that treatment with 1,25-(OH)$_2$D$_3$ or analogs may not always cause growth inhibition and tumor size reduction. It is therefore of utmost importance to identify the mechanism(s) by which 1,25-(OH)$_2$D$_3$ exerts its inhibitory and stimulatory effects on cell growth. This may provide tools to assess whether treatment of a particular tumor will be beneficial. Moreover, purely from a mechanistic point of view, the presence of growth-stimulated and growth-inhibited cells, like the 1,25-(OH)$_2$D$_3$ sensitive and resistant cells, may provide tools to examine the 1,25-(OH)$_2$D$_3$ mechanism of growth regulation.

VII. CONCLUSIONS

The data obtained so far, on (1) the distribution of the VDR in a broad range of tumors and (2) the inhibition of cancer cell growth, angiogenesis, metastasis, and PTHrP synthesis by 1,25-(OH)$_2$D$_3$, all hold promise for the development of treatment strategies based on vitamin D$_3$ use in a wide range of cancers. Moreover, combination of vitamin D compounds with other antitumor drugs, hormones, or growth factors is an important additional therapeutic option. Throughout the last years data have accumulated on the cellular targets and mechanism of action of 1,25-(OH)$_2$D$_3$–induced cancer
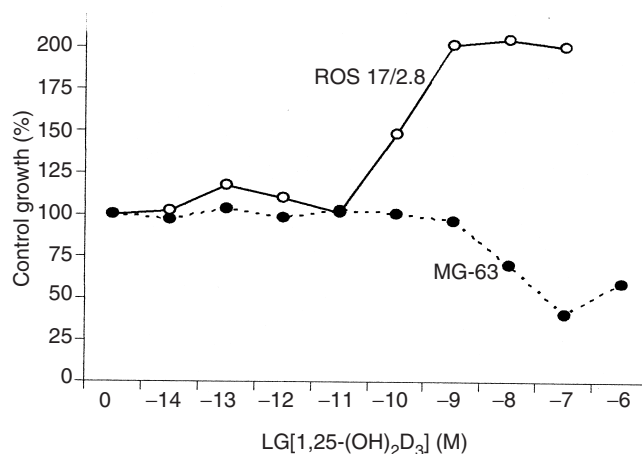
FIGURE 2 Effect of 1,25-(OH)$_2$D$_3$ on proliferation of the osteosarcoma cell lines ROS 17/2.8 and MG-63. Effects on proliferation were examined as described by van den Bemd *et al.* [197].

growth inhibition. The clinical application is enhanced by the development of 1,25-(OH)$_2$D$_3$ analogs with potent growth inhibitory actions and reduced hypercalcemic activity. At the moment more clinical studies are needed in order to firmly establish whether 1,25(OH)$_2$D$_3$ and especially vitamin D$_3$ analogs have therapeutic potential. In the meantime it is crucial to further our understanding of the mechanism(s) by which 1,25(OH)$_2$D$_3$ exerts its effects on tumor cell growth so that these drugs may be employed more effectively.

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Vitamin D, Sunlight, and the Natural History of Prostate Cancer

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I. INTRODUCTION AND BACKGROUND

In 1990, in a paper titled, "Is vitamin D deficiency a risk factor for prostate cancer (Hypothesis)?", Schwartz and Hulka proposed that clinical prostate cancer may be caused by vitamin D deficiency [1]. Since that time, the status of "the vitamin D hypothesis" has gone from that of the proverbial "dark horse" to that of a frontrunner in the race to understand—and to alter—the natural history of this common cancer [2]. This chapter reviews our current understanding of the role of vitamin D in the epidemiology of prostate cancer. We begin with an overview of vitamin D synthesis and of the descriptive epidemiology of prostate cancer.

A. Vitamin D Synthesis

The synthesis of 1,25(OH)$_2$D begins with the production of vitamin D$_3$ (cholecalciferol) after 7-dehydrocholesterol present in the skin is exposed to UV-B radiation (wavelength 290–315 nm). Alternately, vitamin D$_3$ or vitamin D$_2$ (ergocalciferol, a sterol derived from plants) can be obtained from the diet. To become biologically active, vitamin D must undergo two hydroxylations. The first hydroxylation occurs in the liver at the 25th carbon position, forming 25-hydroxyvitamin D (25-OHD), the prohormone and major circulating form of vitamin D. The second hydroxylation occurs at the 1α position, forming 1,25(OH)$_2$D, the hormonal form of vitamin D [3] (see Holick, Chapter 3 for a review of the photobiology of vitamin D). Serum levels of 25-OHD are the best indicator of an individual's overall vitamin D status, whereas serum levels of 1,25(OH)$_2$D are useful in evaluating disorders in calcium and bone metabolism [4].

Classically, the hydroxylation of 25-OHD at the 1α position was presumed to occur exclusively or predominantly in the kidney and the function of 1,25(OH)$_2$D was thought to lie in the control of mineral metabolism. However, it is now clear that local synthesis of 1,25(OH)$_2$D occurs in an autocrine or paracrine fashion in many nonrenal tissues [5], including keratinocytes, colon, and prostate cells [6], where 1,25(OH)$_2$D controls key processes involving cell differentiation and proliferation [7] (see [8] and Hewison and Adams, Chapter 79 for reviews). The discovery of this expanded role for vitamin D in the prostate has important implications for prostate cancer prevention (see Section V).

Sunlight exposure of the skin is by far the most important source of vitamin D [9]. In the U.S., small quantities of vitamin D are added to milk and to some other foods (e.g., margarine, breakfast cereals) principally to prevent rickets. However, vitamin D is not added to milk in many European countries and the quantity of vitamin D in Western diets generally is negligible. It is therefore misleading to consider cholecalciferol and/or ergocalciferol "vitamins," i.e., essential dietary nutrients. They are more accurately conceived of as hormonal precursors and function as vitamins only in the absence of sunlight [10].

B. The Descriptive Epidemiology of Prostate Cancer: Effects of Age, Race, and Place

Prostate cancer is the most common incident (nonskin) cancer among American men and, after lung cancer,

the most fatal, accounting for approximately 221,000 new cases and 29,000 deaths in 2003 [11]. Clinical prostate cancer is strongly age-dependent: mortality rates increase logarithmically with age and are approximately 50% higher among African-Americans than among Caucasians. Other than age and race, the most conspicuous feature of prostate cancer mortality is a striking variation by place: age-adjusted mortality rates vary over twentyfold worldwide, and are highest among African-Americans and northern Europeans [12] (see Fig. 1). The similarity in mortality rates between African-Americans and northern Europeans is an important clue, as it suggests that these populations may share some common factor that underlies their similar mortality experiences. Indeed, whatever theory for the etiology of prostate cancer one proposes, it must answer the question, *How are African-Americans and northern Europeans alike?*
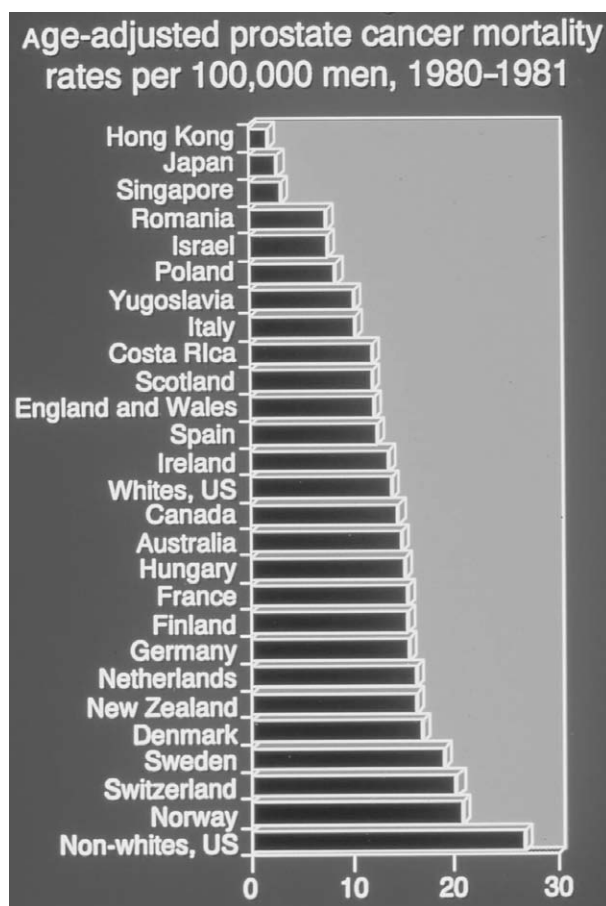
FIGURE 1   Worldwide age-adjusted mortality rates for prostate cancer, drawn from data in Kurihara *et al*. Mortality rates show a twentyfold variation and are highest among African American men and among northern European populations.

A unique feature of the epidemiology of prostate cancer is the high prevalence of "incidental" (also known as "subclinical" or "autopsy") cancer. Autopsies performed on men who have died from causes other than prostate cancer reveal that approximately 27% of men in their 40s and 34% of men in their 50s have histological prostate cancer [13]. The prevalence of these subclinical cancers reaches 60% in men over the age of 80 and continues to increase with age [14]. Histologically, these lesions are indistinguishable from prostate cancers that are potentially life-threatening and are considered to represent cancers at an earlier stage in their natural history. In contrast to mortality rates, the prevalence of incidental prostate tumors is similar among older men worldwide regardless of their racial or geographic origins [15,16]. The discrepancy between the occurrence of clinical and subclinical prostate cancer suggests that clinical cancers result from factors that govern the growth of the subclinical cancers. Thus, any etiologic hypothesis for prostate cancer must also explain why subclinical prostate cancer is ubiquitous, whereas clinical prostate cancer is not.

A related observation concerns the striking effects of migration and of Westernization. In the 1960s, mortality rates for prostate cancer among Japanese living in Japan were 1/15 those of Caucasians in the U.S. These rates quadrupled in first and second generations of Japanese migrants to the U.S. [17]. This suggests either that something in the U.S. promotes the growth of latent cancers among Japanese migrants, or that some factor, more prevalent in Japan than in the U.S., restrains their growth and that upon migration, this restraint is lost. Similarly, age-adjusted mortality rates for prostate cancer among the indigenous Japanese doubled in the time period from 1970 to 1990 [18]. This rapid increase strongly implicates some factor associated with Westernization in the etiology of fatal prostate cancer.

II. PROSTATE CANCER AND THE VITAMIN D HYPOTHESIS

A. Vitamin D and Prostate Cancer Risk

In 1990, we hypothesized that vitamin D maintains the differentiated phenotype of prostate cells and that vitamin D deficiency permits subclinical prostate cancer to progress to clinical disease. Classically, vitamin D "deficiency" had been defined in terms of the bony diseases, rickets (in children) and osteomalacia (in adults). Conversely, vitamin D sufficiency was equated with the absence of bony disease. However, we suggested

that the levels of vitamin D adequate to maintain a healthy skeleton may be inadequate to maintain a healthy prostate.

The vitamin D hypothesis proposed that the major risk factors of the descriptive epidemiology of prostate cancer: increasing age, Black race, and residence at northern latitudes, are related in that each is associated with vitamin D deficiency (see Chapters 47 and 66). Consider the increased risk with age. This is understandable because the prevalence of vitamin D deficiency increases with age. The elderly are commonly vitamin D–deficient for several reasons [19] (see Chapter 50). First, older persons, especially those with limited mobility, often get less solar exposure than younger persons. Second, the thinner epidermis of older individuals contains less 7-dehydrocholesterol than does that of younger individuals and, consequently, less vitamin D_3 is formed following solar exposure [20]. This results in lower serum levels of 25-OHD$_3$ in older individuals. It is now clear that vitamin D deficiency is common among the elderly worldwide, especially for housebound persons and geriatric populations [21]. For example, a recent study of centenarians in Northern Italy revealed that 99 of the 104 persons examined (95%) had undetectable levels of 25-OHD (< 5 nmol/liter) [22].

The higher risk for prostate cancer among Blacks is understandable because black (or otherwise densely pigmented) skin blocks ultraviolet rays, making it more difficult for dark-skinned individuals to synthesize vitamin D from ultraviolet light [23]. Consequently, serum levels of 25-OHD in Blacks are often ½ or less than those of Caucasians living at similar latitudes [24]. It is in this sense that African-Americans "resemble" European populations living at northern latitudes; both groups typically have low serum levels of 25-OHD. Recent population-based data in the U.S. indicate that the prevalence of vitamin D deficiency is ten times higher among African-Americans than among Caucasians [25].

The low risk among Japanese living in Japan is comprehensible as well. Data on serum 25-OHD levels among the Japanese are among the highest ever recorded [26,27]. These high vitamin D levels reflect the traditional diet which is high in oily fish [28]. For example, tuna and skipjack, two of the most commonly consumed fish in Japan, contain approximately 16,000 and 57,000 IU vitamin D per gram of their oil [29]. (For comparison, milk in the U.S. is supplemented with 400 IU vitamin D/quart.) The protective effect of living in Japan would be expected to wane (and clinical prostate cancer rates later to increase) as Japanese migrate and/or adopt a more Western diet.

Table I Risk Factors for Prostate Cancer and Their Interpretation by the Vitamin D Hypothesis, Adapted from Schwartz and Hulka, 1990

Risk factor	Explanation by deficiency hypothesis
Age	The prevalence of vitamin D deficiency increases with age.
Race	
Blacks	Melanin inhibits synthesis of vitamin D.
Asians	Traditional diet high in vitamin D (fish oil) protects against clinical cancer. Protection wanes as migrants adopt a western diet.
Geography	U.S. mortality rates from prostate cancer are inversely correlated with ultraviolet radiation.

This situation is analogous to the recent epidemic of rickets that occurred when individuals from the sun-rich Indian subcontinent migrated to locations in sun-poor northern Europe [30,31].

In summary, the vitamin D hypothesis stemmed from an analogy between the epidemiology of prostate cancer and that of rickets, the "classic" disease of vitamin D deficiency [32]. We reasoned that if vitamin D deficiency could cause one (once-common) disease, it could perhaps cause another, albeit one that presented later in life. Heaney recently labeled such diseases "long-latency deficiency diseases" [33]. In 1990, this hypothesis met with considerable skepticism, as the mechanisms underlying the effects of vitamin D on prostate cells were completely unknown. Although we had predicted the existence of receptors for $1,25(OH)_2D$ (VDR) in human prostate cells (encouraged, in part, by the evidence of VDR in the prostate of the mouse published in 1989 [34]), confirmation of this prediction did not occur until two years later (see Section IV) [35]. The vitamin D hypothesis made many other predictions; in particular, it predicted that, since most vitamin D comes from exposure to sunlight, mortality from prostate cancer should increase as the quantity of available sunlight decreases.

B. Ultraviolet Radiation and Prostate Cancer: Descriptive Studies

In 1992, Hanchette and Schwartz tested this prediction cartographically using data on age-adjusted mortality rates for Caucasian men at the level of the county (Fig. 2) and corresponding data on ultraviolet radiation in the 3,073 counties of the contiguous U.S. We used trend surface analysis, a form of geographic analysis that is essentially linear regression over space [36].
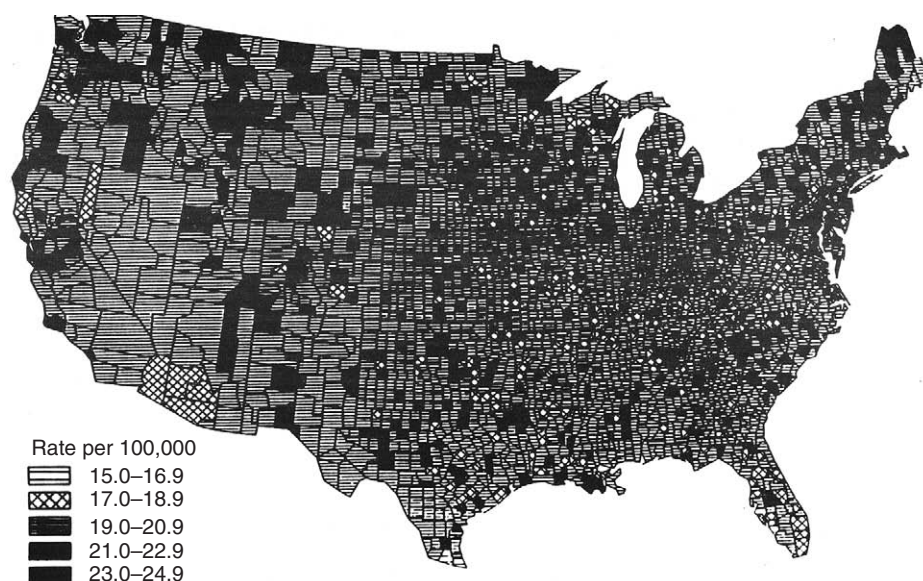
FIGURE 2   Prostate cancer mortality by county among white men, 1970–1979, in the contiguous U.S. Figure from Cancer, 70, No. 12, 1992, p. 2864. Copyright 1992, American Cancer Society. Reprinted by permission of Wiley-Liss, Inc. A subsidiary of John Wiley & Sons, Inc.

Our analyses demonstrated that ultraviolet radiation and prostate cancer mortality among Caucasian men are significantly inversely correlated ($P < 0.0001$) and exhibit opposite geographic trend surfaces [37] (Figs. 3 and 4). The latitudinal gradient in prostate cancer mortality was not apparent in choropleth maps (i.e., the type of maps used most commonly) of prostate cancer mortality (see Fig. 2). These findings subsequently have been replicated using different data sets and analytic techniques for prostate cancer mortality rates in the U.S. [38,39] and Italy [40]. A north-south gradient for prostate cancer also has been reported across Europe [41].

The inverse correlations between the availability of ultraviolet radiation and prostate cancer mortality rates added support for the hypothesis that vitamin D deficiency increases the risk for clinical prostate cancer. However, because these data are based on groups, not individuals, we could not validly conclude that *individuals* with low exposure to sunlight experience lower rates of prostate cancer. (To do so risks committing the "ecologic fallacy," an error in reasoning in which relationships observed at the level of the group are mistakenly applied to individuals [42]). Testing the hypothesis that lower exposure to sunlight increases the risk of prostate cancer in individuals requires epidemiologic studies on individuals (see Section III).

III. OBSERVATIONAL STUDIES

A. Seroepidemiological Studies

Numerous seroepidemiologic studies have attempted to "shed light" on the vitamin D hypothesis. In 1993, Corder *et al.* analyzed data on stored sera from members of the Kaiser Permanente Plan in northern California. Serum levels of 25-OHD and 1,25(OH)$_2$D were measured for 181 men who subsequently were diagnosed with prostate cancer. Although serum levels of 25-OHD were similar among cases and controls, serum levels of 1,25(OH)$_2$D were slightly but significantly lower among cases, with a mean difference of 1.8 pg/ml. The effect was greatest in men over the age of 57 and in men with low serum levels of 25-OHD. Low 1,25(OH)$_2$D levels were associated with palpable and anaplastic tumors, but not with well-differentiated tumors or tumors discovered incidentally at surgery for benign prostatic hyperplasia (BPH).

Subsequently, six studies have addressed the subject of prostate cancer risk in association with circulating vitamin D metabolites. A small case-control study by Braun *et al.* (1995) based on 61 cases failed to find any difference between cases and controls [43]. Gann *et al.* (1996) studied 232 cases of prostate cancer and 414 age-matched controls from participants in the Physicians' Health Study. Median levels of 25-OHD, 1,25(OH)$_2$D
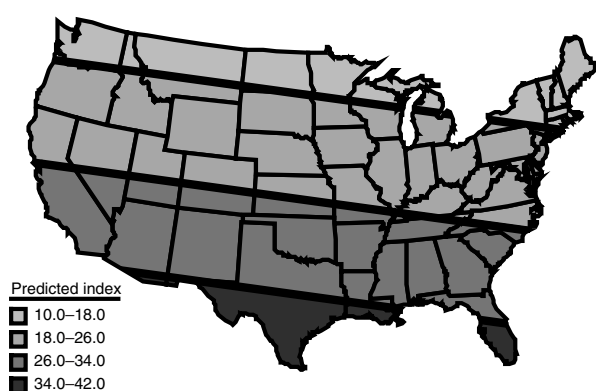
FIGURE 3 Linear trend surface map of ultraviolet radiation in 3,073 counties of the contiguous U.S. Figure redrawn from Cancer, 70, No. 12, 1992, p. 2865. Copyright 1992, American Cancer Society. Reprinted by permission of Wiley-Liss, Inc. A subsidiary of John Wiley & Sons, Inc.

and vitamin D–binding protein were indistinguishable between cases and controls. A nested case-control study conducted by Nomura *et al.* in Hawaii (1998) compared 136 cases to 136 controls and reported that the risk of prostate cancer was reduced, although not significantly, among men with high levels of both 25-OHD and $1,25(OH)_2D$ [44]. Because this study was conducted in Hawaii, very few men had low serum levels of 25-OHD.

Conversely, three larger studies have yielded results in support of the vitamin D hypothesis. Ma *et al.* (1998) examined the associations between serum vitamin D metabolites and polymorphisms in the VDR in relation to prostate cancer risk in the Physician's Health Study [45]. They observed no significant associations overall among
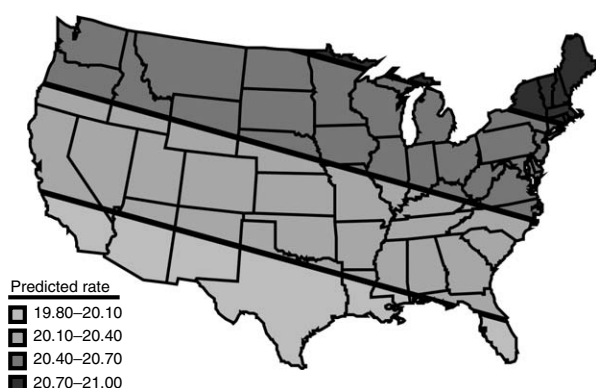
FIGURE 4 Linear trend surface map of age-adjusted prostate cancer among white men, 1970–1979 in 3,073 counties of the contiguous U.S. Figure redrawn from Cancer, 70, No. 12, 1992, p. 2865. Copyright 1992, American Cancer Society. Reprinted by permission of Wiley-Liss, Inc. A subsidiary of John Wiley & Sons, Inc.

372 incident cases and 591 controls for these VDR polymorphisms and prostate cancer. However, in an analysis restricted to men with plasma 25-OHD levels below the median (i.e., among men with relative vitamin D insufficiency), the relative risk for men with the BB vs. the bb genotype was 0.43 (95% CI = 0.19–0.98). This risk reduction was more pronounced among older men (RR = 0.18, 95% CI = 0.05–0.68).

Ahonen and colleagues (2000) conducted a nested case-control study of 19,000 middle-aged Finnish men who attended the first screening of the Helsinki Heart Study and were free of clinical prostate cancer at baseline [46]. After 13 years of follow-up, 149 cases of prostate cancer were identified. Cases were matched to probability-density sampled controls (four per case) and were matched for age, residence, and time of sample retrieval. Prostate cancer risk was inversely related to serum 25-OHD levels at baseline. (Because 25-OHD is the best marker of vitamin D status, levels of $1,25(OH)_2D$ were not measured.) Men with 25-OHD levels below the median (40 nmol/L) had an OR of 1.7 (95% C.I. 1.2–2.6) compared to men above the median, and their mean age at diagnosis of prostate cancer was significantly younger.

Recently, Tuohimaa reported the results of a large, nested case-control study among Nordic men (Norway, Finland, and Sweden). They studied serum 25-OHD levels in 622 prostate cancer cases and 1,451 matched controls. They reported a "U–shaped" risk of prostate cancer, with both low (≤ 19 nmol/L) and high (≥ 80 nmol/L) 25-OHD serum levels associated with higher prostate cancer risk. In the Finnish men, an increased risk was seen for the lowest compared to the highest quintile (OR = 1.9, 95% CI = 0.97–3.7), a result concordant with that previously reported by Ahonen *et al.* for Finns in the Helsinki Heart Study. However, in addition to significantly increased risk at low levels of 25-OHD, the risk of prostate cancer also appeared to increase for men at the highest levels of summertime 25-OHD (compared to normal levels of 25-OHD), especially among Norwegian and Swedish cases.

There are at least two possible explanations for the increased risk seen at high levels of 25(OH)D. As noted by Tuohimaa, high dietary vitamin D may be associated with other risk factors. For example, some dietary sources of vitamin D (e.g., supplements of fish liver oil, commonly consumed in Norway) are also very rich in vitamin A. Vitamin A and its metabolites are known to antagonize the prodifferentiating and antiproliferative actions of $1,25(OH)_2D$ [47,48]. An alternative explanation was proposed by Vieth, who noted that winter at high latitudes produces a gradual decline in serum levels of 25-OHD, and that during this decline the autocrine synthesis of $1,25(OH)_2D$ by the prostate

cannot be maintained. This decline would be greatest in men whose summer levels of 25-OHD were highest [49]. Thus, high levels of 25-OHD *per se* would not increase risk for prostate cancer, but cycles of vitamin D inadequacy would.

In summary, the data from seroepidemiologic studies of 25-OHD and 1,25(OH)$_2$D are conflicting. However, several conclusions can be drawn. First, the findings of Corder *et al.*, of lower levels of 1,25(OH)$_2$D prior to the diagnosis of cancer, have not been confirmed. It is possible that these findings reflect confounding by pre-existing cancer. The average interval between blood draw and diagnosis in the study by Corder *et al.* was 11 years, although in some instances was less than 2 years. Because prostate cancer has a very long natural history (20 years or longer), serum levels of 1,25(OH)$_2$D may have been depressed by pre-existing prostate cancer. Serum levels of 1,25(OH)$_2$D are known to decrease with increasing stage of disease in patients with cancer of the breast [50] and of the colon [51].

Second, although the results from three case-control studies were null, results from the largest prospective studies support the hypothesis that lower serum levels of 25-OHD increase prostate cancer risk. It is noteworthy that 2 of these studies occurred in Scandinavia, locations where vitamin D insufficiency may be common, especially during winter. The finding by Tuohimaa that elevated levels of 25-OHD also were associated with increased risk for prostate cancer among Norwegians and Swedes is an isolated finding and, as these authors note, is not consistent with the larger epidemiologic and biological literature.

In 1998, Giovannucci hypothesized that dietary calcium should increase the risk of developing prostate cancer because calcium acts to reduce serum levels of 1,25(OH)$_2$D [52]. Whether calcium increases prostate cancer risk is unclear: both positive [53,54] and negative studies [55,56] have been reported (see Chapter 91). If calcium does increase risk, the mechanism is unlikely to involve a decrease in serum levels of 1,25(OH)$_2$D since, with the exception of Corder *et al.*, seroepidemiologic studies do not support an association between decreased serum levels of 1,25(OH)$_2$D and prostate cancer [57]. Alternately, calcium may decrease circulating serum levels of 25-OHD. For example, Bell *et al.* showed that the addition of a 2,000 mg/day calcium for 4 days blunted the increase in serum 25-OHD caused by a dose of 100,000 IU/day by 50%, from a 24 ng/ml rise in 25-OHD to only 12 ng/ml [58].

Finally, we note that the seroepidemiologic studies of vitamin D and the risk of prostate cancer have important methodological limitations. One limitation is the use of a single serum sample to estimate vitamin D status. This may be insensitive to detect vitamin D insufficiency. A second is the timing of this sample *vis à vis* the natural history of prostate cancer. The timeframe(s) during which vitamin D protects against prostate cancer is unknown. For example, is it 5–10 years before diagnosis, as might be expected if vitamin D slowed the growth of an existing tumor, or does vitamin D exert effects earlier, perhaps in childhood, as might occur if vitamin D acted as a differentiating agent that altered the eventual phenotype of the adult prostate cell? We have shown that exposure of rats to 1,25(OH)$_2$D at birth dramatically changes the phenotype of the rat prostate in adulthood [59]. If similar phenomena occur among men, then studies that sample vitamin D status in adult life may be measuring vitamin D at a time that is of limited relevance to the natural history of prostate cancer. Studies of lifetime vitamin D exposure, for example, as obtained through residential records and validated solar histories, can be especially informative in this regard (see Section III.C).

B. VDR Polymorphism Studies

The effects of vitamin D in prostate cells are mediated through the VDR, which is a member of the steroid/nuclear receptor superfamily. In target cells, the VDR binds 1,25(OH)$_2$D with high affinity and specificity. The interaction initiates a complex cascade of events beginning with the formation of a heterodimeric complex with the retinoid X receptor (RXR) on specific vitamin D response elements (VDREs) within the promoter region of vitamin D–responsive genes, and influences the rate of RNA polymerase II-mediated transcription of these genes [60] (see Chapters 11 and 13). At present, at least 60 genes including those involved in cell-cycle arrest, apoptosis, and differentiation of prostate cells are known to be regulated by 1,25(OH)$_2$D [61].

The recognition that prostate cells express VDR led to a series of studies of prostate cancer in relation to VDR polymorphisms (see Chapter 68). VDR polymorphisms have been identified in multiple sites including exons 2, 8, and 9 of the VDR gene, which involve Fok I, Bsm I, and Taq I restriction fragment length polymorphisms (RFLPs) (respectively). The Fok I RFLP generates a VDR protein with three additional amino acids at the N-terminus, whereas no coding sequence is affected with Bsm I and Taq I RFLPs. A microsatellite polymorphism in the 3′ untranslated region that does not alter the VDR coding sequence also has been identified. The findings of a number of studies have recently been subjected to a meta-analysis [62].

The first report on VDR polymorphisms and prostate cancer appeared in 1997 by Taylor *et al.* who reported an association between Taq I RFLP and prostate cancer

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risk in men from North Carolina [63]. Subsequently, numerous studies from Asia, Europe, and the U.S. have shown significant associations between prostate cancer and Taq I [64–67] Bsm I [68,69], Fok I [70], and the poly-A microsatellite [71]. Conversely, a similar number of studies have reported nonsignificant or null findings [72–79].

The inconsistencies in these findings may be due to several factors. First, the Bsm I polymorphism is a poor marker for the VDR 3′ untranslated region in some populations [80], and this may explain contradictory findings using this marker. Second, the existence of associations between prostate cancer and VDR polymorphisms may depend upon the stage of prostate cancer examined and upon the choice of the control group. For example, Hamasaki et al. found that the frequency of the TT genotype was significantly higher among prostate cancer patients with locally advanced or metastatic disease when these were compared to controls without cancer (OR=3.52, 95% CI 1.59–7.80; TT vs. Tt/tt) but not when they were compared to controls with BPH [67]. Third, variation in skin types that determine vitamin D_3 synthesis may influence the outcome of these studies [81]. Most importantly, most studies of VDR polymorphisms have been performed in the absence of serum levels of vitamin D and/or in the absence of data on solar exposures. It is possible that, as in the study by Ma et al., VDR polymorphisms convey risk only when 25-OHD levels are marginal or low. Lastly, variability in 1-α-hydroxylase levels in the prostate likely play an important role in vitamin D responsiveness (see below), but these have yet to be investigated in conjunction with VDR polymorphisms.

C. Studies of Sunlight Exposure

As noted above, for most individuals, sunlight is the most important source of vitamin D. A single whole body exposure to a minimal erythema dose of solar radiation can produce approximately 10,000 IU (250 μg) vitamin D_3. Exposure of only parts of the body produces smaller but considerable amounts of vitamin D. For example, Barger-Lux and Heaney calculated that for lightly clad men at northern latitudes in the U.S. (Nebraska, Kansas, and North Dakota) summertime work outdoors was equivalent to a daily oral dosing of 69.5 μg vitamin D_3 (2780 IU vitamin D_3) [82].

Several epidemiologic studies have examined the risk of prostate cancer in relation to exposure to ultraviolet (UV) radiation. Luscombe and colleagues conducted a case-control study of prostate cancer and UV exposure in North Staffordshire, UK. Two hundred ten men with prostate cancer were compared to 155 men with BPH

on various measures of lifetime sunlight exposure. A high sunbathing score was significantly protective for prostate cancer (OR=0.83, 95% CI 0.76–0.89). Conversely, a low exposure to ultraviolet radiation was associated with a significantly increased risk (OR=3.03, 95% CI=1.59–5.78). Interestingly, multiple sunburns during childhood were significantly inversely associated with risk of prostate cancer (OR=0.18, 95% CI 0.08–0.38). Subsequently this group reported that among men with low levels of ultraviolet exposure, those with an inability to tan (skin type 1 of the Fitzpatrick system, "always burn/never tan" [83]) were significantly protected compared to other skin types (types 2–4). They interpreted these data to indicate that among men with low levels of UV exposure, an inability to tan is advantageous because it permits greater cutaneous synthesis of vitamin D [84].

An important methodological issue in retrospective studies such as Luscombe et al. is recall bias. Due to the popularization of the vitamin D hypothesis in the lay press (e.g. [85,86]), many men with prostate cancer may be familiar with this idea and, consciously or not, may under-report their actual exposures to ultraviolet radiation, leading to a bias in support of the hypothesis. Three studies that circumvent this problem are those of Freedman et al. and Robsahm et al., both of which determined ultraviolet exposures using data from death certificates, and John et al., which used data on sunlight exposures that were collected prospectively.

Freedman et al. [87] conducted a death certificate-based case-control study of mortality from prostate in association with residential and occupational exposure to sunlight. Cases were deaths from cancer between 1984 and 1995 in 24 states. Controls were age-frequency matched to cases and were deaths from causes other than cancer and neurological diseases like multiple sclerosis, which are thought to involve sunlight exposure [88]. Occupational exposure to sunlight was classified based on usual occupation listed on the death certificate. Residential exposure to sunlight was classified by state of residence at birth and at death. In order to reduce error from persons with varied solar histories, persons with discrepant places of birth and death (about 25% of the sample) were excluded. These authors found that high residential (but not occupational) exposure to sunlight was associated with a significantly decreased risk of fatal prostate cancer (OR=0.90, 95% CI 0.87–0.93).

Robsahm and colleagues [89] investigated whether variation in ultraviolet radiation influenced the prognosis of prostate cancers diagnosed in Norway. Due to its large spread in latitude (ranging from 58° N to 71° N), Norway experiences large regional and seasonal

differences in ultraviolet radiation [90]. Robsahm *et al.* used death certificate data to measure occupational sun exposure, residential region in one of eight predefined north-south strata, and season of diagnosis for prostate cancer. No differences were observed for occupational exposure or for case-fatality rates in the different geographic regions. However, significant variation in case-fatality rates were seen according to season of diagnosis. Diagnoses made in the summer and fall, when serum 25-OHD levels are highest, were associated with significantly lower case-fatality rates. The authors concluded that vitamin D levels at the time of diagnosis significantly alter prognosis from prostate cancer.

John *et al.* [91] analyzed data from the First National Health and Nutrition Examination Survey (NHANES I) Epidemiologic Follow-up Study in order to test the hypothesis that sunlight exposure reduces the risk of developing prostate cancer. One hundred and fifty-three men with incident prostate cancer were identified from a cohort of 3,414 white men who completed the dermatologic examination and were followed up to 1992. Age-adjusted relative risks (RR) and 95% confidence intervals (CI) were estimated for various measures of sunlight exposure using Cox proportional hazards. The data were adjusted for the confounding effects of education, income, BMI, height, alcohol consumption, smoking, physical activity, energy intake, and intake of fat and calcium. The state of longest residence in the South (RR = 0.58, CI = 0.38–0.88, $p < 0.01$) and high solar radiation in the state of birth (RR = 0.48, CI = 0.30–0.76, $p < 0.01$) were associated with substantial and significant reductions in the risk of prostate cancer. The prospective design utilized by John *et al.* essentially precludes the possibility of recall bias. Moreover, loss to follow up in NHANES I was very low (~5%), greatly minimizing the possibility of selection bias. These findings are consistent with those of Hanchette and Schwartz at the ecologic level, which showed a 50% mortality difference from northern to southern U.S. counties (Figs. 2 and 3). The findings of John *et al.* also are consistent with those of Friedman and colleagues with respect to a protective effect of residential sunlight exposure. The protective effects of solar exposure at place of birth observed by John *et al.* also are consistent with the findings of Luscombe and colleagues that high solar radiation in childhood (i.e., frequent sunburns as a child) exerts a protective effect.

In summary, the data from analytic epidemiologic studies support the hypothesis that exposure to sunlight protects against clinical prostate cancer. Moreover, at least some of this protection appears to occur relatively early in life.

D. Studies of Dietary Vitamin D

Recently, several epidemiologic studies of prostate cancer risk have included information on dietary vitamin D (e.g, Kristal *et al.*, 2002 [92]). The quantity of vitamin D typically observed in these studies is small (less than 400 IU/day). Heaney *et al.* calculated that a dose of 400 IU/d would raise serum levels of 25-OHD$_3$ only modestly, by 7.0 nmol/L (< 3 ng/ml). Given the variability in assays for measuring 25-OHD, this quantity may be too low to significantly alter serum 25-OHD levels reproducibly in individuals [93,94]. Thus, small differences in vitamin D levels, such as those typically associated with Western diets, may be difficult to detect and may be of limited biologic significance with respect to prostate cancer.

Conversely, diets that are high in oily fish can contain appreciable quantities of vitamin D. In this regard, several epidemiologic studies have reported that frequent consumption of fish is associated with a reduced risk of prostate cancer. In a case-control study in the UK, Ewings and Bowie compared 159 cases with prostate cancer to 161 men with benign prostatic hypertrophy and 164 nonurological hospital controls. They found no increased risk associated with dietary fat, sexual activity, and farming—the hypotheses originally under investigation, but found a striking effect of fish consumption (0/159 cases reported fish consumption vs. 14/325 controls, for an "undefined" (infinite) OR, 95% CI = 0.00–0.60) [95]. The Health Professional's Follow-up Study also reported strong inverse associations between fish intake and risk of metastatic prostate cancer. Similarly, a study from the Swedish Twin Registry reported a significant relationship between total fish consumption and prostate cancer mortality [96]. Other positive and negative studies have been reported and are summarized in a recent review [97]. Because the quantity of vitamin D varies greatly among different fishes and even among different parts of the same fish (e.g., livers may contain 100 times the vitamin D$_3$ of skin or viscera) [98], future studies of fish consumption and prostate cancer risk should focus on the type and parts of fish consumed.

IV. EXPERIMENTAL STUDIES OF THE VITAMIN D HYPOTHESIS

By far, the greatest influence of the vitamin D hypothesis has been on laboratory investigations. The effects of vitamin D on prostate cells is the subject of numerous recent reviews [99–103] and is discussed in detail in Chapter 94. Here, we note that these studies demonstrate the presence of VDR in prostate cells and establish that

vitamin D metabolites exert pleiotropic and often profound anticancer effects upon these cells.

The first published laboratory study on the presence of VDR in prostate cancer was performed by Miller and colleagues (1992) and utilized the LNCaP prostate cancer cell line. Although LNCaP cells are immortal (and thus have one essential feature of cancerous cells), they are considered to be a model of well-differentiated prostate cells (e.g., they express androgen receptor and secrete Prostate Specific Antigen). Miller *et al*. demonstrated the existence of functional VDR in LNCaP cells and showed that exposure of these cells to $1,25(OH)_2D$ stimulated their differentiation. This finding, they concluded, "is consistent with the hypothesis of Schwartz and Hulka in that physiological concentrations of vitamin D_3 promote the differentiation of prostatic carcinoma cells." These results have been replicated and extended by many other groups [104–107]. Subsequently, VDR were demonstrated in seven well-characterized human prostate cancer cell lines [108] and physiological levels of $1,25(OH)_2D$ were shown to inhibit their proliferation [104,108]. Moreover, physiological doses of $1,25(OH)_2D$ were shown to inhibit the proliferation of primary cultures of noncancerous human prostate cells [109] and to exert striking anti-invasive and anti-metastatic effects *in vitro* and *in vivo* [110,111]. These findings have led to the active investigation of vitamin D compounds as therapeutic agents in prostate cancer [112], discussed in detail in Chapters 94 and 97.

V. 1,25(OH)₂D IS AN AUTOCRINE HORMONE IN THE PROSTATE

A. 1α-Hydroxylase Is Present in the Prostate

Although laboratory studies confirmed that $1,25(OH)_2D$ inhibits the proliferation, invasion, and metastasis of prostate cancers, a major conceptual problem for the vitamin D hypothesis remained: we had shown that prostate cancer mortality rates are inversely correlated with UV radiation. We interpreted these findings in support of the hypothesis that $1,25(OH)_2D$ maintains the differentiated phenotype of prostate cells and that low levels of $1,25(OH)_2D$ increase the risk for clinical prostate cancer. The conceptual problem was this: Systemic levels of the pro-hormone, 25-OHD, are dependent upon exposure to ultraviolet radiation [113]. However, in normal individuals, systemic levels of the active hormone, $1,25(OH)_2D$, are tightly regulated and are *not* correlated with systemic levels of 25-OHD [114]. Furthermore, although serum levels of 25-OHD are lower among African-Americans than Caucasians, serum levels of the $1,25(OH)_2D$ are not [115]. Thus, how could exposure to ultraviolet radiation result in the exposure of prostate cells to higher levels of $1,25(OH)_2D$?; and, How could the excess mortality from prostate cancer among African-Americans be explained? We reasoned that these problems would be solved if prostate cells synthesized their own $1,25(OH)_2D$ from circulating levels of 25-OHD. We examined this possibility in 1998.

We investigated three well-characterized human prostate cancer cell lines, LNCaP, DU145, and PC-3, and two primary cultures of cells derived from noncancerous human prostates (one normal and one BPH) for their ability to synthesize $1,25(OH)_2D$ (i.e, for evidence of 1α-hydroxylase activity) [6]. The enzymatic reactions were performed in the presence of 1,2-dianilinoethane, an antioxidant and free radical scavenger, and in the presence and absence of clotrimazole, a cytochrome P450 inhibitor. To obtain a definitive answer, the product, $1,25(OH)_2D$, was determined by two different methods. First, we used a thymus receptor binding assay, which specifically recognizes $1,25(OH)_2D$. Second, we utilized radioactive 25-OHD$_3$ as the substrate, and the radioactive $1,25(OH)_2D$ produced was analyzed with a high performance liquid chromatographic (HPLC) system, which is capable of separating $1,25(OH)_2D$ from other vitamin D metabolites and from products of nonenzymatic reactions.

Our data demonstrated clearly that two of the three human prostate cancer cell lines, PC-3 and DU 145 cells, as well as primary cultures of noncancerous prostatic cells, possess 1α-hydroxylase activity. Furthermore, 1α-hydroxylase activity was severalfold higher in the primary cultures from noncancerous prostate tissue than in the prostate cancer cell lines. These data (which were generated prior to the cloning of the 1-α-hydroxylase by several groups in the 1997) subsequently have been confirmed using reverse transcriptase PCR amplification of the 1-α-hydroxylase gene.

B. 25-Hydroxyvitamin D Exerts Antiproliferative Effects on Prostate Cells

The intracellular production of $1,25(OH)_2D$ by prostatic cells suggested that 25-OHD might regulate the differentiation and proliferation of prostate cells. This interpretation would be consistent with the excess prostate cancer mortality observed at higher latitudes and with the excess among African-Americans. We therefore studied the effects of 25-OHD and $1,25(OH)_2D$ on the proliferation of primary cultures of prostatic epithelial cells using [³H]thymidine incorporation into

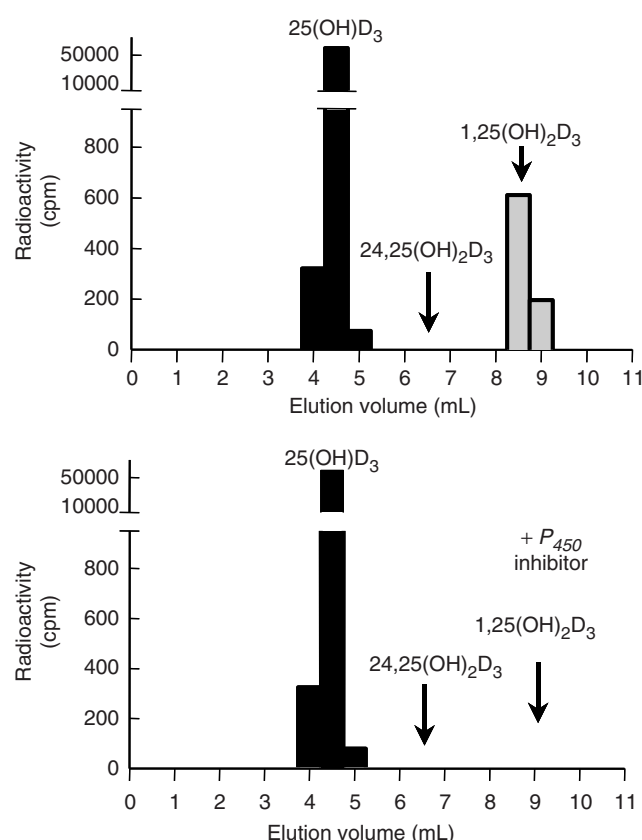FIGURE 5   Human prostate cells in primary culture synthesize $1,25(OH)_2D_3$ from $25(OH)D_3$. HPLC elution profile of tritium activity of lipid extracts from cultured normal human prostate primary cultures incubated with $[^3H]25\text{-}(OH)\text{-}D_3$. Second passage of normal human prostate cells were incubated with nonradioactive 50 nM 25-(OH)-D$_3$, 0.91 µCi/nmol $[^3H]25\text{-}(OH)\text{-}D_3$, and 10 µM DPPD at 37 °C for 2 h in the presence and absence of 20 µM clotrimazole, P450 inhibitor. The lipid extract was applied to a C-18-OH cartridge. The fraction eluted from the cartridge with 6% isopropanol in hexane was dried down under nitrogen and reconstituted in the normal phase solvent containing methylene chloride:isopropanol (19:1). Aliquots of 30 µl of sample plus 10 µl of each 25-(OH)-D$_3$ (100 ng), 24,25(OH)$_2$D$_3$ (100 ng), and 1,25(OH)$_2$D$_3$ (100 ng) as standards (as indicated) were run simultaneously on a 5-µm particle size Econosphere normal phase silica column using 5% isopropanol in methylene chloride at a flow rate of 0.5 ml/min. Fractions collected were dried down under nitrogen, and counted for radioactivity.

DNA and by counting cell number for cell proliferation assays [116] and also by high density growth and clonal growth assays [117]. 25-OHD and 1,25(OH)$_2$D each inhibited growth in a dose- and time-dependent manner. The potencies of 25-OHD and 1,25(OH)$_2$D were not significantly different. Growth inhibition in both the $[^3H]$thymidine incorporation and the clonal assays was evident at 1 nM of 25-OHD. Importantly, the concentrations of 25-OHD used in the assays are

well within the normal physiologic range of 25-OHD in humans (35–100 nM) [118]. These data indicate that 25-OHD, which previously was considered to have little biological activity, can become a potent antiproliferative hormone for prostatic cells that express 1-α-OHase.

Our findings established that there are, in fact, two vitamin D systems in the prostate: an endocrine system in which 1,25(OH)$_2$D is manufactured by the kidney and an autocrine system in which the prostate manufactures its own 1,25(OH)$_2$D. These findings have implications for the interpretation of epidemiologic studies of prostate cancer risk in relation to vitamin D. For example, if prostate cells synthesize their own 1,25(OH)$_2$D *in vivo*, then systemic levels of 1,25(OH)$_2$D may not reflect levels of 1,25(OH)$_2$D at the level of the prostate cell. Thus, the risk of prostate cancer may be influenced by intraprostatic as well as systemic levels of 1,25(OH)$_2$D. These data suggest that risk of prostate cancer may be more closely associated with serum levels of 25-OHD than with 1,25(OH)$_2$D, a finding consistent with the pattern of results that has emerged from seroepidemiologic studies.

The autocrine synthesis of 1,25(OH)$_2$D by prostatic cells also has important implications for the use of vitamin D metabolites in prostate cancer chemoprevention. It is now clear that 1,25(OH)$_2$D exerts pleitropic anticancer effects on normal and cancerous prostate cells. However, 1,25(OH)$_2$D is not suitable as a chemopreventive agent because of the risk of hypercalcemia. Our findings raise the possibility that by increasing the available substrate, supplementation of men with 25-OHD or vitamin D could reduce the risk of cancer by promoting the synthesis of 1,25(OH)$_2$D by prostatic cells. Because the 1,25(OH)$_2$D that is produced within prostatic cells should exert its biological effects within the cell, 1,25(OH)$_2$D would not be released into the systemic circulation and the problem of hypercalcemia would be greatly reduced.

C. 1-α-Hydroxylase Levels Are Lower in Prostate Cancers than in Noncancerous Prostates

The findings that prostate cancer cell lines had less 1α-hydroxylase activity than the primary cultures made us wonder whether a decrease in 1α-hydroxylase activity is a characteristic of cancerous prostate cells in general. Consequently, we compared 1α-hydroxylase activity in cells derived from normal, benign prostatic hyperplasia (BPH) and cancerous prostate tissues by HPLC. A comparison among cells derived from four cancer, two BPH, and three normal prostate tissues
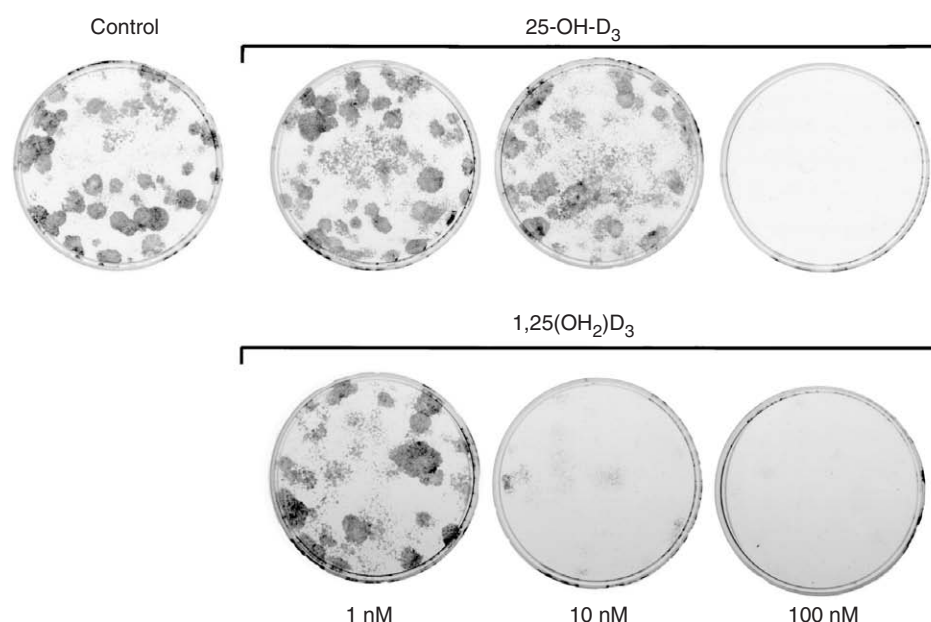
FIGURE 6   Clonal growth of prostatic epithelial cells in response to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. Six hundred cells were inoculated into 60-mm, collagen-coated dishes containing serum-free media with vehicle of the indicated concentrations of 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. After 14 days of incubation, the cells were fixed and stained. Each dose was tested on triplicate dishes. Representative dishes from each treatment are shown. Reprinted from Barretto *et al.*, 2000, with permission.

indicated a marked decrease in 1α-hydroxylase activity in the prostate cancer cells, including an undetectable level of activity in LNCaP cells [119]. This finding has been replicated in a larger sample by Hsu *et al.* (2001) [120]. The differential expression of 1α-hydroxylase activity among these cells is likely regulated at the level of the promoter [121,122]. These findings confirm that loss of 1α-hydroxylase activity, an enzyme that synthesizes the growth-inhibitory hormone, 1α,25(OH)$_2$D, is associated with prostate cancer. However, observational studies cannot determine whether the loss of 1α-hydroxylase activity is a *cause* or a *consequence* of prostate carcinogenesis.

One approach to investigate whether prostate cancer cells are cancerous (e.g., grow independently from normal growth regulatory signals) because they have lost their 1α-hydroxylase activity due to an inability to turn on its gene, is to transfect prostate cancer cells with 1α-hydroxylase cDNA and expression vectors. This should confer antiproliferative activity to 25-OHD in the transfected cells. Since LNCaP cells have little 1α-hydroxylase activity and their proliferation is not inhibited by 25-OHD but is inhibited by 1α,25(OH)$_2$D, we transfected these cells with 1α-hydroxylase cDNA plasmid. Transient or stable transfection of 1α-OHase cDNA into LNCaP cells increased 1α-hydroxylase

activity from undetectable to 4.95 ± 0.69 and 5.8 ± 0.7 pmol/mg protein/hour (respectively). In response to 25(OH)D, transfected LNCaP cells showed a significant inhibition of ^{3}H-thymidine incorporation (37 ± 6 % and 56 ± 4% at 10^{-8} M for transiently and stably transfected cells, respectively). These findings confirm an important autocrine role for 1α,25(OH)$_2$D in the prostate. Furthermore, they suggest that, in conjunction with the systemic administration of 25-OHD, the introduction of the 1α-hydroxylase gene to prostate cancer cells could constitute an endocrine form of gene therapy [123].

D. The Prostatic 1α-hydroxylase Is Not Regulated by PTH or Calcium

The discovery that noncancerous prostate cells possess 25-hydroxyvitamin D-1α-hydroxylase activity raises the possibility that vitamin D or 25-OHD could be used to chemoprevent prostate cancer. However, in order for the prostatic synthesis of 1α,25(OH)$_2$D to be useful in cancer chemoprevention, the prostatic 1α-hydroxylase must not be under the same tight control as is the renal 1α-hydroxylase. We therefore examined whether the prostate 1α-hydroxylase was regulated
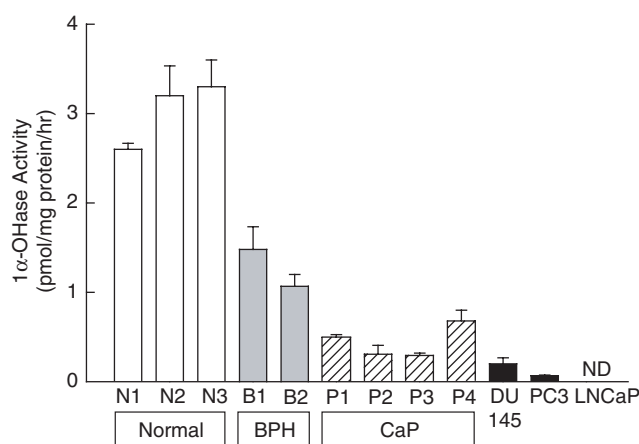
FIGURE 7 1α-OHase activity in primary cultures of normal, BPH, and prostate cancer (CaP), and in human prostate cancer cell lines, DU 145, PC-3, and LNCaP cells. Bars are standard deviations of three determinations. The four primary prostate cultures were obtained from a 63-year-old Caucasian (P1), a 50-year-old African-American (P2), a 67-year-old Caucasian (P3) and a 53-year-old Caucasian (P4) with prostate cancer. Three normal cultures were obtained from histologically normal prostates of a 21-year-old and a 27-year-old donor and a 42-year-old African-American organ donor. BPH cultures were derived from open prostatectomy specimens of a 58-year-old and a 60-year-old Caucasian. Reprinted from Whitlatch *et al.*, 2002, with permission.

VI. VITAMIN D HYPOTHESIS: CONCLUSIONS

Since the vitamin D hypothesis was proposed in 1990, large strides have been made in our understanding of the role of vitamin D in the natural history of prostate cancer. The similarities between the descriptive epidemiology of prostate cancer and vitamin D deficiency have catalyzed investigations in fields as diverse as epidemiology, biochemistry, and experimental therapeutics. Although there is some inconsistency in the observational studies, the results of the larger seroepidemiologic studies, together with the results of studies of sunlight exposure, support the hypothesis that vitamin D insufficiency is causally related to prostate cancer. Experimental studies demonstrate unambiguously that vitamin D metabolites exert prodifferentiating, antiproliferative, antiinvasive, and antimetastatic effects on prostate cells (see Chapter 94). Our discovery that $1,25(OH)_2D$ is an autocrine hormone in the prostate provides the biochemical link between the epidemiologic and experimental data and has opened a new endocrine window on the prostate cell.

The recognition of an expanded role for vitamin D in the prostate suggests roles for vitamin D in many stages of the natural history of prostate cancer. The burden of prostate cancer can be conceptualized as an

by calcium, parathyroid hormone, and $1\alpha,25(OH)_2D_3$, three major regulators of the renal 1α-hydroxylase [124]. Treatment of primary prostate epithelial cells derived from normal prostate tissue for 24 hours with PTH at 10 nM and 100 nM had no significant effect on the 1α-hydroxylase activity. Conversely, enzyme activity decreased to 66 ± 4 and $20 \pm 11\%$ of the control in the presence of 10 nM and 100 nM $1\alpha,25(OH)_2D_3$, respectively. Using the transformed noncancerous PZ-HPV-7 cells (cells that were derived from epithelial cells of the peripheral zone of the normal prostate tissue by transfecting with HPV18 DNA [125]), no significant changes in 1α-hydroxylase activity were observed either at 6 or 24 h after media calcium concentration was changed from 0.03 mM to 1.2 mM with EGF. In contrast, $1\alpha,$hydroxylase activity in HKC-8 kidney cells, used as a positive control, was inhibited 40% in the presence of 1.2 or 2.4 mM calcium. The demonstration that the intra-prostatic synthesis of $1\alpha,25(OH)_2D$ in cultures is unaffected by PTH and calcium confirms that the prostate 1α-hydroxylase is distinct from the renal enzyme. The lack of regulation by PTH and calcium supports the use of vitamin D and 25-OHD as chemopreventive agents for prostate cancer because their administration should cause an increased synthesis of $1\alpha,25(OH)_2D$ within prostate cells.

FIGURE 8   Transfection of 1α-OHase into LNCaP cells confers antiproliferative activity to 25(OH)D. Panel A: Effect of $25(OH)D_3$ (10^{-8}M) on ^{3}H-thymidine incorporation into DNA of LNCaP cells with or without transient transfection with PCR 3.1 vector, anti-sense (AS), or sense PCR 3.1-1α-OHase cDNA (S). Panel B: Effect of $25(OH)D_3$ (10^{-8}M) on ^{3}H-thymidine incorporation into DNA of LNCaP cells stably transfected with vector PCR 3.1 or with sense PCR 3.1-1α-OHase cDNA. Bars indicate the standard deviation of 8 determinations. $^*p < 0.05$. Reprinted from Whitlatch *et al.*, 2002, with permission.
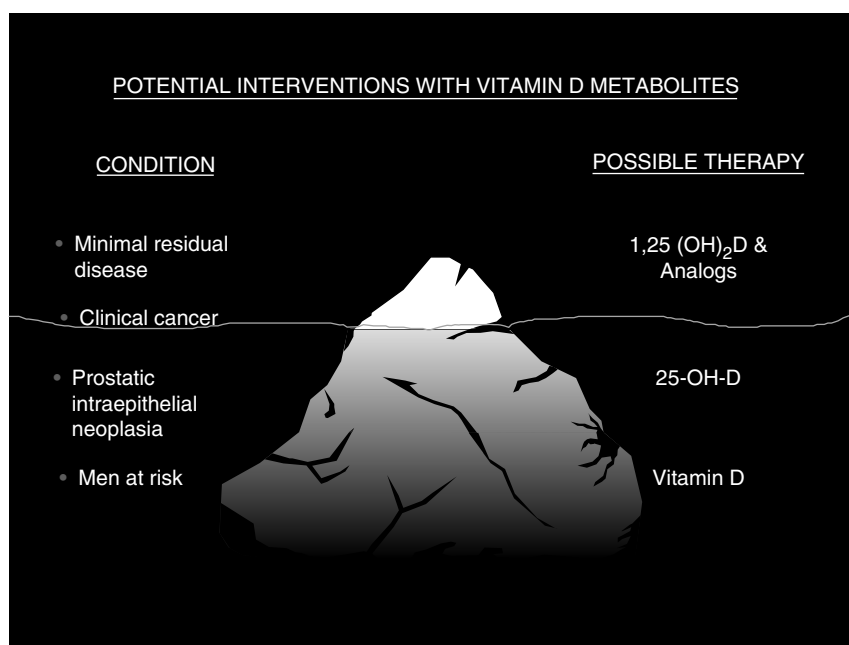
FIGURE 9   The natural history of prostate cancer and possible treatments with vitamin D. At each stage of prostate cancer, there is a corresponding treatment with some form of vitamin D, ranging from the hormonal precursor (vitamin D_3) in men at risk for prostate cancer, to active vitamin D, $1,25(OH)_2D_3$ and its analogs, for men with advanced disease.

iceberg (see Fig. 9). The vast majority of prostate cancers are latent or subclinical (i.e., beneath the water-line in the figure). At the apex of the iceberg are men with clinical disease. These men, especially those men with disease that no longer responds to androgen with-drawal, may be candidates for clinical trials using the hormonal form of vitamin D or its less calcemic analogs. Because clinical prostate tumors appear to have less 1α-hydroxylase than noncancerous prostates, men with clinical cancer are unlikely to benefit from pro-hormonal forms of vitamin D and should be treated with some form of active vitamin D. Men who do not have clinical disease but who are at increased risk for prostate cancer, such as men with histological evidence of prostate intraepithelial neoplasia (PIN), a possible precursor of prostate cancer [126], or men with a pos-itive family history of prostate cancer, may consider supplementation with 25-OHD or vitamin D, as these drugs should be converted to $1,25(OH)_2D$ intraprostat-ically. Finally, for men at the base of the iceberg, i.e., vir-tually all other men, especially men who live at extreme geographic latitudes and/or who receive little effective exposure to sunlight, prophylactic supplementation with vitamin D may be prudent.

In the 20th century, public health programs of vitamin D supplementation virtually eliminated rickets.

Whether vitamin D can reduce the burden of prostate cancer is a challenge for public health in the 21st century.

Acknowledgments

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Epidemiology of Cancer Risk: Vitamin D and Calcium

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I. INTRODUCTION

This chapter will review the epidemiological studies of vitamin D and calcium in relation to risk of colorectal and prostate cancers. Vitamin D physiology is discussed in extensive detail throughout this book, so only a brief introduction to issues relevant for epidemiologic studies is provided here. In epidemiologic studies, two metabolites of vitamin D have been typically measured in the blood, 25-hydroxycholecalciferol (25(OH)D) and 1,25-dihydroxycholecalciferol (1,25(OH)$_2$D). Circulating 25(OH)D is formed in the liver from cholecalciferol, or vitamin D, which itself can be made by photoproduction through ultraviolet light conversion of 7-dehydrocholesterol in the skin [1]. Thus, circulating 25(OH)D concentrations vary with exposure to sunlight, in combination with constitutional factors such as skin pigmentation and aging, which both tend to diminish the capacity for this conversion [2]. In addition to photoproduction in the skin, vitamin D is also contributed by foods, such as fish, eggs, butter, and fortified milk products and breakfast cereals, and vitamin D–containing multivitamins and supplements. Circulating 25(OH)D is the best indicator of nutritional vitamin D status [1]. The hormone 1,25(OH)$_2$D, produced from 25(OH)D in the kidney, enhances calcium absorption from the small intestine and is tightly regulated to ensure calcium homeostasis [3]. Low intakes and low circulating calcium concentrations tend to increase the production of 1,25(OH)$_2$D, which then initiates a series of physiologic actions to stabilize circulating calcium concentrations.

In addition to its mineral regulatory functions, *in vitro* and *in vivo* studies indicate that calcium and vitamin D metabolites, particularly 1,25(OH)$_2$D, participate in the regulation of cellular proliferation and differentiation. These properties have generated the hypothesis that calcium and vitamin D may have some anticancer properties in humans, particularly for colorectal and prostate cancer. Although 25(OH)D is the best indicator of nutritional status of vitamin D, generally the anticancer properties have been attributed to 1,25(OH)$_2$D. However, recent evidence suggests that other tissues, including the prostate [4] and colon [5] besides the kidney can convert 25(OH)D to 1,25(OH)$_2$D, thus raising the possibility that 25(OH)D can have direct anticancer effects itself in addition to being the precursor to 1,25(OH)$_2$D. While clearly our understanding of potential anticancer mechanisms has increased dramatically recently, the role of calcium and vitamin D in the prevention of human cancer remains unsettled. Results from *in vitro* or short-term intervention studies based on presumed intermediate endpoints such as proliferation do not lend to simple predictions for cancer risk associated with long-term moderate differences in calcium intake or vitamin D levels. Thus, epidemiologic studies remain an important component of our understanding.

Over the past several decades, a number of epidemiologic studies for colorectal and prostate cancer have provided important data concerning the vitamin D and calcium hypothesis. Our knowledge in this area has improved, but many questions remain because of various methodologic limitations. The types of studies used

have been those that have examined sunlight exposure as a determinant of vitamin D synthesis in the skin, studies of vitamin D intake and of calcium and dairy food intake, studies of circulating vitamin D metabolites, studies of polymorphisms in the vitamin D receptor, and rarely intervention studies. This chapter will critically review the studies of calcium and vitamin D in relation to colorectal and prostate cancers, the cancers that have been most studied, focusing on strengths and weaknesses of the methodology. Recommendations for future studies will be made.

II. COLORECTAL NEOPLASMS

A. Colorectal Cancer

Colorectal cancer is one of the most common cancers in developed countries. In the United States, approximately 105,500 cases of colon cancer and 42,000 of rectal cancer cases are expected to occur in 2003 [6]. Colorectal cancer is the third leading cause of cancer deaths in each sex and second for both sexes combined, ultimately afflicting approximately 6% of individuals in their lifetime. Approximately half of individuals diagnosed with this disease will die from it. Factors related to a "westernized" lifestyle and dietary pattern, including obesity, sedentary lifestyle, and "western" diet pattern are believed to be among the most important factors related to the etiology of colorectal cancer [7]. Over the past two decades, two additional, though not entirely independent, hypotheses have been that higher calcium intake and higher circulating vitamin D metabolites are associated with a lower risk of colorectal cancer. The evidence for these hypotheses is reviewed here. The effects of Vitamin D on colon cancer cells are discussed in Chapter 95.

1. SUNLIGHT EXPOSURE

The vitamin D hypothesis for colorectal cancer originated over two decades ago when Garland and Garland [8] observed that the states with the highest mean solar radiation, such as New Mexico and Arizona, had the lowest rates of death from colon cancer, whereas states with the lowest mean solar radiation, including New York, New Hampshire, and Vermont, had the highest mortality rates of colorectal cancer. The authors hypothesized that the higher mortality rates of colorectal cancer in the less sunny regions were caused by inadequate vitamin D status (see Chapter 90). After this report, additional studies that have evaluated the hypothesis of a relation between colorectal cancer incidence [9,10] or mortality [11] and solar radiation [9,10] or ultraviolet light-blocking air pollution [11] have generally supported the vitamin D hypothesis. These studies encompass cancer rates in

entire populations, but relatively little data exist for sunlight exposure at the individual level in case-control or cohort studies. One exception is a case-control study of 1993 colon cancer cases and 2410 population controls interviewed in Northern California, Utah, and Minnesota that did not find a relationship with reported sunshine exposure [12]. Evidence on sun exposure and cancer is reviewed further in detail in another chapter.

2. CASE-CONTROL AND COHORT STUDIES OF VITAMIN D INTAKES

The relationships between colorectal cancer and dietary or supplementary vitamin D were investigated in cohort studies of men [13,14] and women [15–17] or both sexes [18,19], and in case-control studies [12,20–26]. Some studies did not support an association, but more than half suggested inverse associations [13–16,19,21,23,25,26]. Among the studies supporting an inverse association, the relative risk (RR) for colorectal cancer ranged from 0.88 comparing extreme quintiles of intake of dietary and supplemental vitamin D in the Nurses' Health Study, which consisted of 501 cases among 89,448 women followed for 12 years [16], to 0.5 comparing extreme quartiles of vitamin D intake (P trend < 0.05) in the Western Electric study, which consisted of 47 cases among 1,924 men followed for 19 years [13]. A Swedish population-based case-control study of 352 colon and 217 rectal cancers noted a moderate inverse association (extreme quintiles odds ratio (OR=0.5, 95% CI 0.3–0.9) between rectal cancer only and dietary vitamin D (supplements not assessed) in multivariate, but not age-adjusted analysis [25]. In the Iowa Women's Study, women in the highest tertile of intake had a moderately lower risk of rectal cancer (OR=0.76; P trend=0.20) [17]. In a recent cohort study based on the large Cancer Prevention Study II Nutrition Cohort [19], total vitamin D intake from diet and supplements was inversely associated with risk of colorectal cancer (RR=0.71, 95% CI 0.51–0.98). Many of these studies controlled for some confounding factors, but almost all dietary vitamin D comes from fortified milk products, fatty fish, and multivitamin supplements, so conclusively attributing the benefit to vitamin D alone is difficult. For example, much of the vitamin D comes from dairy products, which also are the major sources of another putative protective factor, calcium.

3. STUDIES BASED ON PLASMA MEASURES OF VITAMIN D

The studies summarized above have examined vitamin D exposure based on surrogates of sunlight exposure or reported dietary intakes. Epidemiologic studies have not considered simultaneously dietary

vitamin D and vitamin D contribution from sunlight exposure as direct exposures, but three studies have measured circulating vitamin D concentrations, which integrate dietary sources and endogenous production of vitamin D. Two of the studies were based from the Washington County, Maryland cohort. The first study from this cohort included 34 cases with a lag of months to eight years between blood donation and colorectal cancer diagnosis, and 67 controls. In this study, an inverse relation between 25(OH)D and colon cancer was observed, although the relation was somewhat attenuated in the highest quintile [27]. The second study based on this cohort included 57 cases with 10 to 17 years between sample collection and time of diagnosis and 114 controls; in this study, neither 25(OH)D nor $1,25(OH)_2D$ was related to colorectal cancer risk [28]. The other study was nested in the Finnish Alpha-Tocopherol, Beta-carotene Cancer Prevention Study. In that study, no relation was observed between serum $1,25(OH)_2D$ concentration and colorectal cancer among the 91 colon and 55 rectal cancer cases and 290 controls, but an inverse relation was suggested for 25(OH)D level, particularly for colorectal cancer (extreme quartiles: OR=0.3, 95% CI 0.1–1.1, P trend = 0.04) [29]. Thus, the epidemiologic data are limited, but do suggest an inverse association with 25(OH)D. These results are of interest considering recent evidence of 1 α-hydroxylase activity in the large bowel mucosa [5].

B. Colorectal Adenoma

Colorectal adenomas are well-established precursor lesions for the majority of cancers [30]. Thus, these have been used as intermediate endpoints for colorectal neoplasia and to examine risk factors for early stages of carcinogenesis. The relation between sunlight exposure and adenoma risk has not been studied.

1. DIET-BASED STUDIES OF VITAMIN D

Six case-control studies [24,31–35] and two cohort studies (published in one report) [36], and one study of recurrent adenomas [37] have examined the relation between vitamin D and the risk of colorectal adenoma. Of these studies, only two suggested an inverse association between vitamin D intake and adenoma risk [24,36]. Furthermore, findings were not consistent across gender or by location within the colon. In one of the case-control studies, which consisted of 154 small and 208 large adenoma cases and 426 polyp-free controls, an inverse association between vitamin D intake was seen only for small adenoma in women (extreme quintiles: OR=0.4, P trend = 0.04) [24]. In the cohort studies, an inverse relation was seen only for rectal

adenoma in women using the baseline dietary assessment and eight years of follow-up (extreme quintiles: RR=0.30, 95% CI 0.13–0.71, P trend = 0.005), but not a subsequent dietary assessment and the latter four years of follow-up [36]. Thus, overall the results on vitamin D intake and risk of adenoma are not consistently supportive of an association.

2. STUDIES BASED ON PLASMA MARKERS OF VITAMIN D

In the past several years, some studies have examined plasma vitamin D levels and adenoma risk. These studies examined distal colorectal adenomas, and no data are available for proximal adenomas. One study of 473 cases with an adenoma and 507 controls with no adenoma at sigmoidoscopy measured only 25(OH)D [33]. The results from this study suggested an inverse, but not statistically significant association with 25(OH)D (OR for high versus low quartile = 0.74, 95% CI 0.51–1.09). Another study also examined only 25(OH)D with risk of colorectal adenoma among 239 cases and 228 controls among patients who had had sigmoidoscopy [34]. In this study, each 10 ng/mL increase of serum 25(OH)D was associated with a 26% decrease in risk of adenoma (OR=0.74, 95% CI 0.60–0.92). The lower risk was primarily in the deficiency range. Another study, the only one that was prospective, was nested in the Nurses' Health Study and examined both $1,25(OH)_2D$ and 25(OH)D in 326 matched case-control pairs [38]. Overall, mean plasma 25(OH)D and $1,25(OH)_2D$ were only slightly nonsignificantly lower in cases than controls. However, women whose plasma $1,25(OH)_2D$ was below 26.0 pg/mL, a level typically considered to be below normal, were at increased risk of distal colorectal adenoma (OR=1.58, 95% CI 1.03–2.40). Plasma 25(OH)D displayed a U-shaped pattern, with risk lowest in the middle two quartiles. The data are sparse, but are consistent with an inverse association with 25(OH)D and possibly with $1,25(OH)_2D$.

C. Polymorphisms in the Vitamin D Receptor Gene

The effects of $1,25(OH)_2D$ are mediated by its binding to the vitamin D receptor (VDR), which transactivates transcription of target genes [39]. A number of polymorphisms in the VDR have been identified (see Chapter 68). These include: a poly-A microsatellite in the 3′-untranslated region, and the following restriction length polymorphisms: BsmI in intron 8 (denoted b), ApaI in intron 8 (denoted a), and TaqI in exon 9, which results in a base, but not amino acid change (C352T and denoted t). All of these polymorphisms are in strong linkage disequilibrium. None of these polymorphisms

affects the amino acid coding sequence of the vitamin D receptor, although the BAt haplotype has been associated with greater vitamin D receptor transcriptional activity or enhanced mRNA stability in artificial gene constructs than the baT haplotype. In addition, individuals possessing the BB genotype have higher circulating $1,25(OH)_2D$ levels than those with the Bb or bb genotypes [40,41], although the mechanism is unknown.

Another polymorphism in the VDR has been identified in exon 2 by using the FokI enzyme. The presence of the FokI site (f allele) results in a VDR that is 427 residues long, whereas the absence of this site (F allele) results in a shorter VDR of 424 residues [42]. The F allele has been associated with higher bone mineral density [43] and lower risk of bone fractures [44]. Thus, the activity of the VDR related to the F allele is believed to be greater than that related to the f allele.

One study has examined VDR variants in relation to colon cancer risk and three in relation to adenoma risk. In a case-control study of 250 population-based cases of colon cancer and 364 controls, the BAt haplotype (i.e. BBAAtt vs. all others) was related to a lower risk of colon cancer (OR = 0.5, 95% CI 0.3–0.9) [45]. As expected because of their being in linkage disequilibrium, each of the individual genotypes (B,A,t) was similarly related to lower risk. The FokI variant was not related to risk (OR = 1.0, 95% CI 0.6–1.8 for ff versus FF). The adenoma study described above [34] that found an inverse relation with 25(OH)D did not find an association with the FokI variant, although risk was nonsignificantly lower for the FF variant (OR = 0.75, 95% CI 0.36–1.58 for FF versus ff). In contrast, another study of 373 colorectal adenoma cases and 394 controls found that compared to the FF genotype, the OR was 0.79 (95% CI 0.44–1.41) for the Ff genotype and 0.32 (95% CI 0.11–0.91) for the ff genotype [46]. In a clinic-based case-control study of 393 and 406 controls, compared with the bb genotype, neither the Bb (OR = 0.86, 95% CI 0.63–1.19) nor the BB genotype (OR = 0.77, 95% CI 0.50–1.18) was appreciably associated with adenoma risk [35]. However, as in the only study on colon cancer described above [45], the BB genotype was in the direction of lower risk.

D. Discussion: Vitamin D and Colorectal Neoplasia

The potential influence of vitamin D in relation to colorectal cancer risk has been examined in a variety of ways. Studies based on sunlight exposure first led to the vitamin D hypothesis because individuals living in sunnier regions tend to have a lower risk of colorectal

cancer mortality [8]. Although provocative, interpretation of ecologic studies as indicative of a causal relation between colorectal cancer and vitamin D is limited because excluding potentially confounding factors is difficult due to lack of individual-specific exposure information. For example, migration from one region of the country to another prior to time of cancer diagnosis or death, time spent outdoors by season of the year, shading by buildings, use of sunscreen, amount of skin covered by clothing, and skin pigmentation all influence photosynthesis of vitamin D [47] (see Chapter 3). Ecologic studies cannot control for potentially confounding factors; for example, outdoor physical activity, a protective factor for colorectal cancer, or regional variations in red meat and alcohol consumption, which are risk factors for colorectal cancer [7]. An approach that has not been utilized much is the examination of region or sunlight exposure in case-control or cohort studies, which in addition to measuring region, sunlight exposure, and mediating factors such as skin color, could also adjust for potential confounders such as dietary practices that may differ across regions. Finally, indirect measures of exposures at the population level may sometimes give false impressions. For example, in one study that sampled elderly individuals throughout Europe, residents in Northern Europe had circulating 25(OH)D concentrations twice as high as similarly aged individuals living in Southern Europe [48]. This apparent paradox may be explained by the higher milk intake in the northern regions. Thus, the data based on sunlight exposure are interesting and suggestive, but studies focusing on sunlight exposure need to be enhanced with more rigorous study designs.

A second line of evidence has been based on studies that have examined dietary intake of vitamin D in relation to risk of colorectal cancer or adenomas. The results based on cancer risk have been mixed and perhaps weakly supportive, whereas those on adenomas have been less supportive. There are many important considerations for these studies. First, studies based solely on dietary intakes, even assuming that vitamin D intake is measured adequately, would still substantially misclassify overall vitamin D status because of lack of information of sunlight exposure. Second, some degree of measurement error is inherent in the measure of vitamin D intake, and the degree of measurement error is not typically quantified in studies. Third, most studies measure intake at one point, and the presumed time relation between vitamin D status and risk of colorectal neoplasia is not well established. Fourth, in most populations studied, high intake of vitamin D represents a combination of high intake of (fortified) dairy products, fatty fish, and multivitamin supplements.

Because other components of these have been hypothesized to reduce risk of colorectal cancer: calcium (dairy products), omega-3 fatty acids (fish), and folic acid (multivitamin supplements), even if an inverse association between vitamin D intake and colorectal cancer risk is observed, the possibility for confounding by other factors is difficult to exclude. Although the dietary studies of vitamin D are moderately supportive at best, many factors could attenuate true associations; thus, the data do not exclude an important effect of dietary vitamin D.

Perhaps the strongest approach to evaluate the vitamin D hypothesis is to examine plasma levels of vitamin D. However, in total, the relevant studies are comprised of just over 200 total cases of colorectal cancer. Recently, more data have been available for adenomas. The use of a biomarker has some theoretical advantages. Presumably, a biomarker can integrate the influence of sunlight exposure, dietary intake, and constitutional factors. However, a potential limitation is that most studies rely on a single measurement of $1,25(OH)_2D$ or $25(OH)D$. Substantial intra-individual variability could exist; for example, simply the season the blood sample is taken could strongly influence an individual's vitamin D level. While season could be matched or adjusted for in analyses, seasonal variation will add heterogeneity; for example, a measurement in the winter will not reflect how high vitamin D levels may rise in the summer for an individual. Another limitation of available studies is that the vitamin D–binding protein has not been studied; variation in this binding protein could be an important determinant of bioavailable vitamin D. To date, the evidence is much too sparse and conflicting to make a definitive statement for either circulating $25(OH)D$ or $1,25(OH)_2D$ and colorectal neoplasia.

With the identification of VDR genotypes, another approach to study the relation between vitamin D and risk of colorectal cancer has emerged. This tool has been used in relatively few studies, but may prove fruitful if several conditions are met. First, the functionality of the known and future identified polymorphisms must be established. Second, studies must be well designed and sufficiently large to examine moderate-sized effects. Third, concurrent measures of vitamin D status ideally should be available. Early and limited results are consistent with a possibly lower risk of colorectal neoplasia associated with the BAt haplotype, but more study is needed. Only a few studies have considered the FokI polymorphism and these are conflicting.

In summary, the vitamin D hypothesis for colorectal cancer remains viable, and some epidemiologic data are intriguing. Although the data in each area of study are not compelling on their own, it is noteworthy that some data regarding sunlight exposure, dietary vitamin D, plasma vitamin D, and VDR polymorphisms are supportive of the vitamin D hypothesis. However, the full range of available epidemiologic tools has been hardly utilized. Future large studies that incorporate data on dietary and supplement intake, sunlight exposure, plasma levels of vitamin D metabolites, and VDR polymorphisms are likely to provide a much stronger test of this hypothesis.

E. Calcium

Although calcium interacts with the vitamin D axis, the notion of a role of calcium against colorectal cancer originated independently of this relationship with vitamin D [49]. The major hypothesis has been that calcium reduces colorectal cancer risk by binding secondary bile acids and ionized fatty acids to form insoluble soaps in the large bowel lumen [50,51]. If unbound, these compounds are purported to irritate the colorectal mucosa, causing proliferation and repair processes that could be carcinogenic. The role of the vitamin D receptor in this respect is discussed in Chapter 53. Alternatively, calcium was proposed to directly alter colorectal mucosal activity [52,53]. This section deals with the epidemiologic studies of calcium and colorectal neoplasia.

1. ECOLOGIC STUDIES

Ecologic studies have suggested an inverse relation between intakes of calcium [54,55], milk, and other dairy products, the primary dietary calcium source [56,57], and death from colon or rectal cancer. However, the selection of specific populations for the comparisons may have biased the estimate. In contrast to these studies, when a wider array of populations are chosen, a slight positive association between per capita milk and milk product consumption and colon cancer mortality emerges [58]. One exception to this pattern is Finland, where consumption of calcium is high but rate of colon cancer is relatively low. Ecologic studies have important limitations for this association because milk and dairy consumption tend to be high in westernized or economically developed countries, which may have a higher risk for other reasons, including obesity, sedentary lifestyle, and other dietary factors [7]. It is plausible that despite the lack of an inverse correlation across countries (or even a positive association), calcium or milk intake at the individual level could be protective among individuals in a high-risk population. In fact, in one analysis, the correlation between per capita milk and milk product intakes and age-adjusted

colon cancer mortality was 0.40, but after adjustment for per capita animal fat, the correlation reversed to –0.30 [56]. Thus, overall, because of the limitations of the ecologic studies and the alternative interpretations of the results of these studies, these studies provide neither strong evidence for or against a role of calcium in colon cancer risk.

2. CASE-CONTROL AND COHORT STUDIES OF COLORECTAL CANCERS

The association between calcium intake and risk of colorectal cancer has been studied in a number of case-control and cohort studies over the past two decades. Among the case-control studies [12,21–25,59–74], the majority [12,22,23,60,61,64,65,68–71,73–75] suggested a protective effect of calcium on colorectal cancer risk, but only in about half [12,22,23,61,64,68–70,73,74] were the findings statistically significant overall, or at least in one subsite or gender. Two of these studies found statistically significant inverse associations between calcium intake and colon [70] and colorectal [61] cancer only in women. In a large case-control study of Chinese men and women and North American Chinese, an inverse relation for calcium was noted for the rectum only in the North Americans [68], while in a case-control study in Italy, an inverse relation was observed for the colon, but not the rectum [23]. A recently published meta-analysis [76] concluded that the results of case-control studies are heterogeneous and on the whole do not provide evidence of an association between total intake of dairy products, milk, cheese, or yogurt and colorectal cancer risk. Although the meta-analysis did not examine explicitly calcium intake, the data are not strongly supportive of the calcium hypothesis because dairy products are the major source of calcium. However, of note, substantial heterogeneity exists, and a good number of the individual studies are consistent with a moderate protective influence.

The data from cohort studies have been more supportive of an association [13–20,75,77–82]. Of these, most [13–15,18,19,75,77,79–82] suggested an inverse association between calcium intakes and colorectal cancer risk, although the inverse association was statistically significant in only some of these studies [13,19,77,81,82]. The meta-analysis that did not support a role for dairy products in case-control studies did show a statistically significant inverse association for total dairy products (OR = 0.62, 95% CI 0.52–0.74, and for milk (OR = 0.80, 95% CI 0.68–0.95) in the cohort studies [76]. Notably, no heterogeneity of the risk estimates in the cohort studies was evident.

The meta-analysis did not include four recent supportive studies for calcium [18,19,81,82], which warrant further discussion. Two of the studies were conducted in Finland [18,81] and two in the U.S. [19,82], including a cohort study combining two populations in the United States (thus, in essence, these represent five distinct study populations) [82]. The study by Jarvinen *et al.* was relatively small, with only 38 cases in the colon and 34 in the rectum. A suggestive inverse association was observed for milk and calcium only for colon cancer but not for rectal cancer. In the study by Pietinen *et al.*, also in Finland, an inverse association was observed for calcium; risk was 30–40% lower in quartiles 2–4, compared with the lowest quartile, which had a median calcium intake of 856 mg/day. A similar pattern was observed for milk. In the study by Wu *et al.* [82], an inverse association was observed only for distal colon cancer both for men of the Health Professionals Follow-Up Study and women of the Nurses' Health Study. Interestingly, men with low intakes of total calcium were at higher risk, but the incremental benefit of additional calcium beyond approximately 700 mg/day appeared to be minimal, suggestive of a threshold effect. In the study by McCullough *et al.* [19], risk also seemed to level off around the range of 1000 mg/day or so, and no additional benefit was observed for intakes exceeding 1200 mg/day. In both U.S. studies, an independent beneficial effect of dietary and supplemental calcium was observed. In the study by Wu *et al.* [82], supplemental calcium was related to lower risk only if dietary calcium was relatively low, supporting both the existence of a threshold effect and a true effect of calcium rather than another factor in dairy products.

3. STUDIES OF COLORECTAL ADENOMA

Some studies have examined calcium intake in relation to adenoma risk. The general design of these studies was to examine the prevalence of adenoma discovered at endoscopy among individuals having a colonoscopy or sigmoidoscopy for diverse reasons. Controls were individuals who did not have an adenoma at endoscopy. Most studies have not observed a relation between calcium intake and adenoma risk [24,31–36,83]. In some studies, the null relation did not vary by source of calcium [36], site within large bowel [36], polyp size [24,36], or gender [24,36]. However, one study of men and women undergoing colonoscopy in North Carolina and consisting of 236 cases of adenomas (27 of which were adenocarcinomas) and 409 controls suggested that calcium intake from diet and supplements was inversely related to adenoma among men, although the trend was not statistically significant (extreme quartiles multivariate OR = 0.44, CI 0.15–1.24); among women no clear relation was evident [84]. In the study by Peters *et al.* a borderline significant inverse association was observed [34].

Two observational studies examined risk of recurrent adenomas in individuals within randomized trials examining other factors [37,85]. In the study by Martinez *et al*. [37], dietary calcium intake of >1068 versus <698 mg/day was associated with an OR of 0.56 (95% CI 0.39–0.80, P trend = 0.007), and for total calcium >1279 versus <778, the OR was 0.62 (95% CI 0.42–0.90, P trend = 0.005). The study by Hyman *et al*. [85] showed only a modest inverse association (OR = 0.72, 95% CI 0.43–1.22) for high versus low quintiles of intake, but found a strong trend for deceasing number of adenomas (P trend = 0.005) across increasing calcium intake. The generally supportive results for recurrent adenomas in observational studies are interesting in light of the results from intervention studies of recurrent adenomas, which are discussed next.

F. Human Intervention Studies of Calcium Intake and Colorectal Adenoma

The hypothesis that calcium reduces risk of colorectal neoplasia was tested in randomized intervention trials. The strongest support of the hypothesis was from an intervention trial of calcium supplementation (1200 mg of elemental calcium vs. placebo) among 913 participants who previously had colorectal adenomas and were then followed for new occurrences of adenomas (sometimes called *recurrent adenomas*) [86]. In that study, the investigators found a moderate but statistically significant reduction in risk of adenoma recurrence: the recurrence rate was 31% in the calcium group and 38% (RR = 0.76, 95% CI 0.60–0.96) in the placebo group. Similar results were observed in the European Calcium Fibre Polyp Prevention trial, a smaller study conducted in Europe around the same time [87]. In that study, the RR for recurrent adenomas was 0.66 (95% CI 0.38–1.17), although the lower recurrence rate among the calcium group was not statistically significant (P = 0.16) in this relatively small study. Together, these data support that higher calcium intake may reduce the rate of new adenomas by about 25 to 35%. The results are consistent with those from the two observational studies of recurrent adenomas. Most critically, these studies provide support of the calcium hypothesis from randomized data, which are not prone to biases that may occur in observational studies.

G. Discussion

1. Interpretation of Evidence

The interpretation of the human studies that examined calcium intake in relation to colorectal cancer and risk is quite complex. The apparently modest association (approximately 15–30% reduction in risk) strains the detection capacity of epidemiologic methods. In fact, case-control studies of cancer and studies of prevalent adenomas, with a few exceptions, tend to be null. The prospective data for cancer as well as for adenoma recurrence and the randomized data for adenoma recurrence tend to support a modest inverse association. The supportive studies are based on prospectively collected data, which tend to be less prone to methodologic biases possible in case-control and prevalent adenoma data, and on randomized data, which is less susceptible to confounding than are observational data. Also, indirectly supportive of a role of calcium are the results of the meta-analysis that showed an inverse association for total dairy products (OR = 0.62, 95% CI 0.52–0.74) and for milk (OR = 0.80, 95% CI 0.68–0.95) in the prospective studies [76]. Dairy products and milk are the primary source of calcium, although other components of dairy products (including vitamin D) could contribute to the benefit. However, the most recent largest prospective epidemiologic studies, which included three of the large prospective studies of diet in the U.S. (Nurses' Health Study, Health Professionals Follow-Up Study, American Cancer Society Cohort) found a benefit with supplemental calcium [19,82].

The randomized data on recurrent adenomas are crucial because they provide evidence that calcium directly influences a colorectal neoplastic endpoint in a setting in which confounding is essentially precluded. The epidemiologic data using the cancer endpoint are complementary because they indicate a similar effect on cancer as observed for recurrent adenomas. Alone, the data on recurrent adenomas would be less compelling because only a small fraction of adenomas (around 10%) progress to cancer, and not all colorectal cancers arise from adenomas. Calcium has a relatively small influence (about a 15–30%) reduction in adenoma risk. If, for example, this association was preferential for adenomas that are less likely to progress to cancer (e.g. small tubular adenomas), then the quantitative impact on cancer could be much less than the results observed in the randomized trials. However, the associations for the cancers suggest a similar, if not stronger, magnitude of association. Thus, the results for recurrent adenomas on the whole are probably generalizable to cancers.

2. Mechanism

The most widely cited hypothesis regarding a benefit of calcium is that calcium might reduce colon cancer risk by binding secondary bile acids and ionized fatty acids to form insoluble soaps in the colonic lumen,

thereby reducing the proliferative stimulus of these compounds on the colonic mucosa [50]. While appealing, a weakness of this hypothesis is the lack of definitive evidence that secondary bile acids and ionized fatty acids underlie the higher risk of colon cancer in Western populations. Although fat is a promoter in animal models of carcinogenesis [88], and production of bile acids and fatty acids is greater with consumption of high fat diets, epidemiologic studies have not found that calcium is more beneficial among people who consume high fat diets [14,16,19,25,59,77,82]. In addition, animal studies find that the protective effect of calcium is not completely dependent on fat intake [49]. A meta-analysis of cohort and case-control studies on calcium and colorectal cancer risk published between 1980 and 1994 found large heterogeneity in estimates not accountable by differences in endpoint, subsite, gender, fat intake, calcium source, and dose, or study design [89].

In recent years, stronger evidence has converged in supporting the role of energy imbalance, obesity, and physical inactivity, rather than fat intake, as being the major "Western" factors related to colon cancer risk [7]. These factors are believed to ultimately increase cell proliferation in the large bowel mediated through insulin and insulin-like growth factors [90]. Interestingly, Ma *et al*. [91] found that calcium intake was related to lower risk of colorectal cancer primarily in individuals with high circulating IGF-1 concentrations. The insulin/IGF-1 hypothesis may coincide with the hypothesis that calcium exerts a direct influence on proliferation. The studies that have examined the influence of calcium intake on proliferation in colorectal crypts have been inconsistent, with some showing a reduction [52,53,92–94], and others not [95–100]. Of those studies that were randomized and placebo-controlled [94–96, 98–100], only one showed a benefit of calcium supplementation [94]. One of the best conducted studies showed no change in the proportion of proliferating cells in the crypt overall (i.e., labeling index or LI) in rectal biopsies from patients with sporadic adenoma supplemented daily with 1 or 2 g for six months, but it did demonstrate a statistically significant reduction in the proportion of proliferating cells in the top 40% of the crypt [98]. Bostick *et al*. suggest that their findings are most compatible with the hypothesis that calcium directly affects progression through the cell cycle, rather than an indirect effect through binding of bile acids and fatty acids.

More recent evidence, reviewed by Lamprecht and Lipkin [49], indicates that the protective effect of calcium involves interactions with vitamin D acting on a series of signaling events at various tiers of colonic cell organization. In a recent study, fasting levels of 25(OH)D but not 1,25(OH)$_2$D, were inversely associated with whole crypt labeling index and the size of the proliferative compartment [101]. Calcium supplementation influenced this relationship between 25(OH)D and proliferative parameters. These data suggest that there may be a local influence of 25(OH)D through conversion of 25(OH)D to 1,25(OH)$_2$D [5].

3. Implications and Future Research

While the apparent protective effect of calcium on colorectal cancer risk appears relatively modest in magnitude, this effect could have important public health ramifications because of the high prevalence of colorectal cancer. For example, approximately 150,000 cases of colorectal cancer are diagnosed annually in the U.S. If, for example, calcium deficiency contributed to even only 10% of these cases, then potentially about 15,000 cancer cases and 7,500 deaths could be prevented annually through the relatively simple dietary intervention of increasing calcium intake. However, resolving the dose-response is critical. Some of the recent large prospective studies find a threshold effect, with most of the benefit arising through avoiding low intakes (e.g., below 500–700 mg/day). Benefits appear to plateau around 700–1000 mg/day, and whether any benefit occurs beyond 1,000 mg/day or so of calcium remains unclear. The randomized trials did not address this because they examined only one dose. Another important issue to resolve is whether vitamin D is a modifying factor of the apparent benefit of calcium. As summarized above, the data on vitamin D and colo-rectal cancer are suggestive, but not definitive. Finally, whether some highly susceptible individuals could be identified, such as those with high IGF-1 levels or a genetic marker, would be important to establish.

III. PROSTATE CANCER

A. International Patterns of Mortality and Incidence

Prostate cancer mortality rates around the world vary more than thirtyfold. The lowest rates are observed in the Far East and on the Indian subcontinent, and the highest rates observed in Western Europe, Australia, and North America [102]. Adjusting the rates to the World Health Organization world standard population, the mortality rate in the year 2000 for prostate cancer was approximately 1 per 100,000 men annually in China compared to 17.9 per 100,000 in the U.S. [103]. The contrast in rates is even higher for prostate incidence: in China the rate is 2.9 per 100,000 men

compared with 107.8 and 185.4 per 100,000 men in white and black Americans, respectively [103]. A sharp gradient in the prostate cancer mortality rate is observed between Northern Europe (e.g., Sweden, Norway, and Denmark), where the rates are more than 23 per 100,000 men annually, and Southern Europe (e.g., Greece), where the rates are half that [102]. Some of this wide disparity in prostate cancer incidence rates among countries is likely due to differences in medical practice leading to differential rates of detection of subclinical tumors. The frequency of these latent tumors does not vary substantially across populations [104,105], indicating that factors that cause the growth and progression of prostate tumors most likely account for the marked variation in prostate cancer incidence. Widespread screening using prostatic-specific antigen (PSA) has greatly increased the numbers of preclinical prostate cancers diagnosed. Many dietary hypotheses have been proposed to explain this variation in rates or progression of prostate cancer, but none have been definitively established. The role of vitamin D in regulating prostate cell growth is discussed in Chapter 94.

B. Vitamin D

1. Sunlight Exposure

Schwartz and Hulka have hypothesized that vitamin D protects against prostate cancer based primarily on correlations between regional UV radiation and prostate cancer mortality rates in the U.S. [106]. Hanchette and Schwartz [107] examined the geographic distributions of UV radiation and prostate cancer mortality in 3073 counties in the U.S., and found a prostate cancer mortality gradient, with rates higher in the North and lower in the South. More recently, Grant [108] examined UV-B data for July 1992 and cancer rates in the U.S. between 1970 and 1994 and confirmed these findings for prostate cancer (as well as many other cancer sites). Luscombe *et al.* [109] conducted a case-control study of 210 cases and 155 controls (with benign prostatic hyperplasia) in the UK and examined whether indicators of UV exposure at the individual level were associated with risk of prostate cancer. They found that childhood sunburn frequency (OR = 0.18, 95% CI 0.08–0.38), regular foreign holidays (OR = 0.41, 95% CI 0.25–0.68), sunbathing score (OR = 0.83, 95% CI 0.76–0.89) and low exposure to UV radiation (OR = 3.03, 95% CI 1.59–5.78) were associated with the risk of prostate cancer. In addition, cases with low UV exposure developed prostate cancer at a younger median age (67.7 years) than cases with a higher exposure (72.1 years). These results were confirmed in another population by

the same group [110]. In addition, these investigators also found that the risk may be modified by skin pigmentation and genes that influence it [111]. These findings provide for the first time some confirmation of the UV hypothesis at the individual level. These provocative results need to be confirmed in prospective analyses to preclude the possibility of recall bias, although the relatively strong associations observed would tend to argue against such bias. The sunlight hypothesis is discussed further in Chapter 90.

2. Dietary Vitamin D

Surprisingly little data has evaluated whether dietary or supplemental vitamin D is related to the risk of prostate cancer, and the limited evidence is not supportive. In the Health Professionals Follow-Up Study, a large cohort of U.S. men [112], no relation was observed between dietary and supplemental vitamin D with intakes ranging from <150 to >800 IU/day and total or advanced prostate cancer. This analysis was based on 1369 cases of prostate cancer. A case-control study of prostate cancer conducted in Sweden also did not support an effect of dietary vitamin D [113]. In addition, a cohort study of Finnish male smokers did not find any association between dietary vitamin D and risk of prostate cancer [114]. A recent case-control study of 605 prostate cancer cases diagnosed at younger ages cancer found no association with dietary vitamin D [115]. As discussed above, the study of dietary and supplemental vitamin D is prone to many limitations. In any case, currently no epidemiologic data support the hypothesis that an increase in dietary vitamin D would lower risk of prostate cancer.

3. Circulating Vitamin D

Six case-control studies nested in prospective cohort studies have examined circulating vitamin D metabolites in relation to risk of prostate cancer [116–121]. Corder *et al.* [116] measured $1,25(OH)_2D$ and $25(OH)D$ levels in blood collected prior to diagnosis in 181 prostate cancer cases and 181 controls nested in the Kaiser-Permanente cohort. Risk of prostate cancer, especially in older men, was significantly lower among those with higher $1,25(OH)_2D$ concentrations. In men over 57 years, compared to the lowest $1,25(OH)_2D$ quartile, the prostate cancer odds ratios were 0.66, 0.53, and 0.37 in the second, third, and fourth quartiles, respectively. Men with the lowest risk of prostate cancer, and particularly of tumors of advanced stage and high grade, were those with high circulating $1,25(OH)_2D$ and low $25(OH)D$.

A much weaker (nonsignificant) inverse relation between overall prostate cancer and prediagnostic plasma, $1,25(OH)_2D$ concentration was observed among

232 cases and 414 age-matched controls nested in the Physicians' Health Study [118]. This study was extended to 372 cases and 591 controls with subsequent follow-up [41]. However, some findings were observed in subgroup analyses that are noteworthy because they were similar to those found in the Corder *et al*. study [116]. When considering jointly $1,25(OH)_2D$ and $25(OH)D$ levels, reduced risks of prostate cancer were noted for high $1,25(OH)_2D$ and low $25(OH)D$ levels in the Corder *et al*. study [116] and in the Gann *et al*. study [118], particularly for older men and for more aggressive prostate cancer, but the joint association was statistically significant only in the Corder *et al*. study [116]. In the Physicians' Health Study, results for vitamin D metabolites also indicated some potentially interesting interactions with VDR polymorphisms (see below).

Three studies were essentially nonsupportive of the vitamin D hypothesis. A study by Braun *et al*. which also evaluated $1,25(OH)_2D$ concentration in blood obtained prior to cancer diagnosis, did not show an inverse relation between prostate cancer and serum $1,25(OH)_2D$ level [117]. This study consisted of only 61 cases, only 34 were nonincidental findings, and only 19 of these were diagnosed in men older than 57 years. Thus, this study had very low power to examine risk of the more aggressive prostate cancers in the older age groups. A study in Hawaii by Nomura *et al*. [119] generally was nonsupportive of the vitamin D hypothesis. An additional study was recently conducted for 460 prostate cancer cases in the Health Professionals Follow-up Study who were diagnosed through 1998 after providing a blood specimen in 1993/95 [121]. Plasma $1,25(OH)_2D$ and $25(OH)D$ concentrations were compared to an equal number of age-matched men. In this study, no association was observed between prostate cancer risk and prediagnostic plasma concentrations of $1,25(OH)_2D$ or $25(OH)D$.

Only one plasma-based study was conducted outside the U.S. This study [120] did not measure $1,25(OH)_2D$, but did show a 1.7-times higher risk of prostate cancer (95% CI 1.2–2.5) in Finnish men whose plasma $25(OH)D$ concentrations were below the median compared to at or above. The association was strongest in men who were younger at study entry (<52 years old: OR=3.1, 95% CI 1.6–6.1) [120]. All the other studies measured $25(OH)D$ and none supported an association between this metabolite and prostate cancer risk [116–119,121]. The authors of the Finnish study indicated that more than half of their participants had $25(OH)D$ levels consistent with clinical vitamin D deficiency [120]; their median of 40 nmol/L (16.0 ng/mL) was near the usual cutpoint for vitamin D deficiency of approximately 15 ng/mL. In the other studies, the

percentages of controls that had $25(OH)D$ lower than 15 ng/mL were ~5% [117], 6.5% [118], 11% [121], and 13.3% [116]. The proportion with vitamin D deficiency was not available in the Nomura *et al*. study, which was conducted in Hawaii, but it is likely low given that their median $25(OH)D$ concentration was 41 ng/mL in the cases and 41.6 ng/mL in the controls [119]. Thus, a possible explanation for the inconsistent findings for $25(OH)D$ and prostate cancer among studies is that in individuals with adequate vitamin D levels there is no added benefit of higher circulating levels, but that an incremental increase in $25(OH)D$ to sufficiency may reduce risk.

An additional factor that has not been studied adequately is the vitamin D–binding protein, which could influence the bioavailability of vitamin D metabolites (see Chapter 8). In the only prospective study that examined this factor, measuring the binding protein did not confer much additional information [118]. One small case-control did suggest those with higher binding protein concentrations may receive less benefit from vitamin D [122]; however, this study was limited by a small sample size, retrospective collection of information and use of convenience controls.

In summary, only one of five studies that measured $1,25(OH)_2D$ found a statistically significant inverse association with prostate cancer risk [116], and one of six that measured $25(OH)D$ found a significant inverse association [120]. Some potentially notable tendencies, but hardly consistent, were that vitamin D is more relevant for the older onset cases, for the more aggressive endpoints, when $1,25(OH)_2D$ is high and $25(OH)D$ is low, and when $25(OH)D$ is in the deficiency range. If vitamin D is more important for progression, as indicated by associations with more advanced endpoints in some studies, then more recent studies in which cases are predominantly PSA-detected may miss an important effect on progression.

C. Polymorphisms in the Vitamin D Receptor Gene and 1-α-hydroxylase

As described above, a number of polymorphisms in the vitamin D receptor have been identified. The following polymorphisms have been evaluated in relation to prostate cancer: a poly-A microsatellite in the 3′-untranslated region, and the following restriction length polymorphisms: BsmI in intron 8 (denoted b), ApaI in intron 8 (denoted a), and TaqI in exon 9 (denoted t). These polymorphisms are in strong linkage disequilibrium, and none of these polymorphisms affects the amino acid coding sequence of the vitamin D receptor [40]. Also, examined in several studies is the

FokI polymorphism in exon 2. The BAt haplotype has been studied relatively extensively for prostate cancer in U.S. and European populations and in Asian populations (mostly in Japan and one study in China). See Chapter 68 for an extensive discussion of the VDR polymorphisms.

For the BAt haplotype, 10 studies have been conducted in the U.S. or Europe. Three of the studies found a statistically significant lower risk associated with the BAt haplotype [123–125]. The largest study, and the only prospective nested case-control study, was the study within the Physicians' Health Study by Ma *et al*. [41]. This study found only a weak nonsignificant lower risk associated with the BAt haplotype, but did find a strong inverse association in subgroups of older men, and those with 25(OH)D below the median. Other studies were nonsupportive [126–131], although three of these studies were seriously limited by small sample sizes (<100 cases) [126,127,131]. Four Japanese studies examined the BAt haplotype. Two, similar to some of the studies in Western populations, found a statistically significant reduced risk associated with the BAt haplotype [132,133]. The three null studies were limited by small number of cases [134–136].

In summary of the studies examining the BAt haplotype, five found a statistically significant inverse association of prostate cancer with the BAt haplotype. The largest study did not, but subgroup analyses offered some support that this haplotype is associated with lower risk, particularly for more aggressive cancers [41]. Overall, the relative risks ranged from two- to fivefold. Most of the null studies had severe power limitations. All of the studies, except the study by Ma *et al*. [41] relied on convenience control groups. Because of the limitations of these studies and the inconsistencies, the conclusions drawn need to be tempered. Nonetheless, the data are suggestive of a lower risk, particularly of aggressive cancers, associated with the BAt haplotype. These results are of interest because the BAt haplotype has been reported to have greater vitamin D receptor transcriptional activity or enhanced mRNA stability in artificial gene constructs than the BAt haplotype, and individuals with the BB genotype may have higher circulating 1,25(OH)$_2$D levels than Bb or bb genotypes [40,41].

Only three studies have examined prostate cancer risk in relation with the FokI genotypes [129,137,138]. The results are inconsistent. In addition, no association has been observed between polymorphisms in the gene encoding 25-hydroxyvitamin D 1-α-hydroxylase, the enzyme that catalyzes the conversion of 25(OH)D to 1,25(OH)$_2$D [139], and prostate cancer risk. Given the limited research, more needs to be done for the FokI

and 1-α-hydroxylase genotypes before even tentative statements can be made.

D. Calcium and Dairy Products

Relevant to the vitamin D hypothesis is intake of calcium and dairy products, which are the greatest source of calcium and of vitamin D in populations that fortify milk with vitamin D. Until just recently, few studies had examined the relation between dietary and supplemental calcium intake and risk of prostate cancer, although more studies have examined associations with milk and dairy consumption. The general pattern has been in stark contrast to that observed for colon cancer: studies of calcium, milk, and dairy products generally show that these are positively related to prostate cancer risk.

This increased risk of prostate cancer associated with higher milk and dairy consumption was first observed for ecologic studies. Countries with greater per capita milk consumption have higher national prostate cancer mortality rates (r = 0.66). This correlation is greater than for other foods high in animal fat (e.g., meats, r = 0.39) [140], suggesting that dairy products impart an adverse effect beyond that of total dietary fat or saturated fat. Milk (r = 0.75) and cheese (r = 0.69) consumption were correlated with regional mortality rates in Italy, even when controlling for other factors characteristic of each region [141,142]. In a more recently conducted ecologic study, based on mortality rates from 41 countries, the nonfat portion of milk had the highest association with prostate cancer mortality rates of dietary factors examined (r^2 = 0.73; P < 0.001) [143]. Although these ecologic studies are consistent with the hypothesis that higher intakes of milk and dairy products are correlated with higher risk of prostate cancer mortality, as noted earlier, these studies are limited by lack of information on individual-specific exposures, outcomes, and confounding factors. Thus, evaluating the relation of sources of calcium in the diet with prostate cancer in case-control and cohort studies is important.

In case-control studies, men consuming high levels of milk and other dairy products have been observed to be at an either statistically significant increased risk [115,144–150], or borderline significant (P ≤ 0.1) increased risk of prostate cancer [151–154] in most studies. One study reported an association with saturated fat from dairy products [155]. Several studies, however, have not supported an association [156,157], and no study has suggested a protective effect. Overall, among the case-control studies, nine support a statistically significant association with some component of

milk or dairy products, four found nonsignificant but borderline significant associations, and two found no associations. The case-control studies are limited because total energy intake was not controlled in most of them, and they have some potential methodologic limitations, such as the potential for recall and selection biases. Nonetheless, they provide strong evidence against a benefit of milk or dairy products, and the positive association observed in many settings (including diverse ethnic groups in the U.S., Italy, Canada, Sweden, Uruguay, Greece) is noteworthy because methodologic limitations and confounding is unlikely to be uniform across such diverse settings.

Most prospective cohort studies, which tend to be less prone to methodologic limitations than case-control studies, also tend to support an association between higher intake of milk or dairy products and risk of prostate cancer [158–162]. However, some have not supported an association [114,163–165]. No studies support a protective influence of dairy products. In the case-control studies reporting an elevated risk, the magnitude of the relative risk comparing high to low consumption of milk is around 2 (ranging up to five-fold) [147]. The associations tend to be weaker for prospective studies, with RRs generally in the range of 1.3 to 1.6 between high and low intakes. The weaker associations in prospective studies might reflect biases that exaggerated the magnitude of the association in case-control studies. Alternatively, more of the prospective studies were conducted more recently when the case mix is heavily skewed toward early-stage, relatively indolent lesions, and some studies had long time lags between the dietary assessment and the diagnosis of cancer. If dietary factors are primarily important for disease progression, then weaker associations would be expected to occur in the cohort studies.

Many fewer studies have examined calcium intake in relation to prostate cancer risk. The effect of calcium intake on prostate cancer risk was directly evaluated in a case-control study in Sweden [113] and in a cohort study in the U.S. [112], with both reporting a positive relation. The cohort study, which consisted of over 50,000 health professionals, found substantially greater associations between calcium intake with metastatic and fatal prostate cancer. Supplemental (12% of the men were calcium supplement users) and dietary (mostly dairy) calcium sources were independently associated with increased risk of advanced prostate cancer. The risk of metastatic prostate cancer was 5 times greater among men consuming >2000 compared to <500 mg/day of calcium from the diet and supplements. An association was also observed in the prospective Physicians' Health Study [161]. Another case-control

study found a twofold increased risk of regional/distant prostate cancer associated with higher calcium intake, but no increased risk with local disease [115]. A recent large prospective study found that very high calcium take (>2000 mg/day) was association with an increased risk of prostate cancer [166]. Interestingly, the association was stronger for men not tested by PSA, indicating an effect on progression. Other studies are nonsupportive [114,162,167,168] or only suggestive of an effect of calcium [153,154].

In summary, from several study designs in a variety of populations, the finding of a positive relation of milk and dairy products with prostate cancer, particularly advanced disease, has been observed frequently though not invariably. Evidence from some recent studies showing a positive association between calcium from supplements and advanced prostate cancer supports an effect of calcium apart from its co-occurrence with fat or other components in dairy products.

E. Discussion

The human evidence on vitamin D, calcium, and dairy products in relation to prostate cancer risk is quite complex to interpret. Some evidence is moderately supportive of a benefit of vitamin D. Both ecologic and a few case-control studies suggest that greater sunlight exposure is associated with a reduced risk of prostate cancer. The studies of VDR polymorphisms are somewhat suggestive, though inconclusive, that differences in VDR might influence risk. Although the functionality of these polymorphisms remains in question, the presence of an association with the VDR genotypes points to the importance of vitamin D. The plasma-based studies are generally nonsupportive but are suggestive of a possible protective role of high circulating $1,25(OH)_2D$, or a risk for deficiency in $25(OH)D$ in the etiology of advanced prostate cancer. Dietary vitamin D studies are limited but nonsupportive. Vitamin D–binding protein concentration is another factor that may modify risk, but the only prospective study did not support a role for this factor; more studies, however, are clearly needed.

An additional complexity in prostate cancer epidemiologic studies is that the recent widespread use of PSA has almost eliminated the presence of advanced disease, at least at initial diagnosis. Additionally, if vitamin D influences progression of the disease rather than early stages, null associations in recent studies where the case mix is dominated by early stage, relatively indolent lesions may not necessarily be considered strong evidence against the vitamin D hypothesis.

In future studies, total prostate cancer as the main outcome is unlikely to be an adequate phenotype to study; better indicators of progression or of aggressive behavior are paramount to identify. In addition, some data, though not all, indicate that some modifiable factors may be more relevant for prostate cancers that have an older age-of-onset. Prostate cancer is a uniquely heterogeneous malignancy, so its study is particularly complex and usually does not allow for simple answers.

The frequent finding of a positive association between intake of milk, dairy products, and possibly calcium and risk of prostate cancer has been enigmatic. Milk and dairy products are the major sources of calcium and dietary vitamin D (in the U.S.) so this finding appears paradoxical given the other evidence that vitamin D exposure could inhibit carcinogenesis. An attempt to reconcile these apparently conflicting observations was the postulation that high intakes of dairy products, despite providing vitamin D, may actually suppress $1,25(OH)_2D$ production [112]. This hypothesis was directly evaluated in the Physicians' Health Study, which had shown an increased risk of prostate cancer associated with higher intakes of skim milk, dairy products, and calcium from dairy products [161]. In that study, men who consumed >600 mg calcium per day from skim milk had a mean plasma $1,25(OH)_2D$ concentration of 30.06 pg/mL compared to men who consumed ≤150 mg/day, who had a mean $1,25(OH)_2D$ concentration of 35.64 pg/mL (P = 0.005). This study was the first to show simultaneously that dairy calcium is associated with a lower $1,25(OH)_2D$ and with an increased risk of prostate cancer. An alternative hypothesis is that the increased risk of prostate cancer often seen with higher dairy product consumption is due to some component of the fat content of dairy products; this is a plausible and not necessarily mutually exclusive hypothesis. However, some studies support an equally strong if not more pronounced association with skim/low fat milk than with whole milk [112,115,143,148,161,165].

Another potentially important recently identified factor is *in vitro* evidence that human prostate cells can synthesize $1,25(OH)_2D$ from 25(OH)D [4] and that 25(OH)D may inhibit the proliferation of primary prostatic epithelial cells [169]. These findings need to be verified *in vivo*, but suggest that high circulating 25(OH)D would decrease risk of prostate cancer. Although the studies based on sunlight exposure would support this, this hypothesis is difficult to reconcile with the generally null studies on dietary and supplementary vitamin D, the weak findings for plasma-based studies of 25(OH)D, and the frequent finding of a positive association with dairy products.

IV. CONCLUSION

In conclusion, the hypothesis that the vitamin D axis plays an important role in carcinogenesis is viable and an active and exciting area of research. Several lines of human and animal evidence support a role for calcium and vitamin D for colorectal cancer. Simple answers are precluded for prostate cancer because of inconsistencies in studies. Clearly, a better understanding of the complex biology that accounts for interactions among dietary factors, UV light, genetic polymorphisms in the VDR and among the vitamin D metabolites will be required to better make sense of the existing epidemiology and to design new studies. Other factors, such as the influence of vitamin D on the insulin-like growth factor axis will have to also be taken into account. Furthermore, effects are likely to differ among the organ sites, as the recent divergent associations for calcium and dairy products for prostate and colorectal neoplasms show. Future epidemiologic studies must be well-designed to avoid methodologic bias, be sufficiently large, and take into account simultaneously factors such as diet, UV exposure, plasma levels, and genetic polymorphisms. This will require large studies and the pooling of data to get more definitive results. In addition, the complexity of the changing prostate cancer case mix cannot be ignored.

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Differentiation and the Cell Cycle

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I. INTRODUCTION

In general, cell cycle control is extremely well-conserved throughout the eukaryotic species. The basic machinery consists of several cyclin-dependent kinases and cyclins that pair with each other, sometimes changing partners, to drive the cell towards and through mitosis [1–4]. This basic arrangement seems almost monotonously similar in all cells, yet in multicelluar organisms control of cell proliferation must be, and is, cell type specific. The required control is provided by proteins that regulate the kinase activity of Cdk/cyclin pairs, most often in a negative fashion, and usually occurs in response to cues from the environment. Importantly, cell cycle changes are also triggered in cells undergoing differentiation.

Differentiation can be considered to be, in essence, a persistent pattern of expression of previously dormant genes, which results in new functional capabilities of the differentiated cell. The new functions require cellular resources that compete with and finally titrate out the resources required for proliferation, and allow an accumulation of negative regulators of the cell cycle (e.g., p27Kip1 [5]), which then predominate over the positive regulators. Thus, there is a reciprocal relationship between cellular differentiation and cell cycle progression/proliferation [6–8], though there is also evidence that differentiation and cycle arrest need not be strictly coupled [9–12].

Cell cycle changes in differentiating cells do not always take place immediately—in some cells there is at first a boost of proliferation—as in normal hematopoiesis, or in HL60 [11–13] and U937 [14] cells differentiating in response to derivatives of vitamin D$_3$.

However, even in these cases, there is an eventual slow-down of the cell cycle traverse and cessation of proliferation of differentiated cells. Consequently, numerous attempts are being made to exploit the differentiating actions of vitamin D derivatives ("deltanoids") to induce proliferative quiescence of neoplastic cells, and thus increase the range of options for optimal therapy of human cancer. This is addressed in other chapters, primarily Chapter 97.

The term "differentiation" is often used in a rather specialized way to imply the acquisition of new functional properties by a cell that already appears to be mature and capable of function. Examples are monocytes "differentiating" into macrophages, or naive T lymphocytes becoming helper or cytotoxic cells. Little is known, however, of cell cycle alterations in this form of differentiation.

II. INDUCTION OF DIFFERENTIATION BY 1,25(OH)$_2$D$_3$ AND ANALOGS ("DELTANOIDS")

A. Principal Models

In 1981 the Suda Laboratory reported that an *in vitro* exposure of M1 mouse myeloid leukemia cells to 1,25(OH)$_2$D$_3$ induces these immature cells to differentiate into functional macrophages [15]. This seminal finding was followed by numerous reports not only confirming that leukemic cells, murine or human [16–19], differentiate in response to treatment with 1,25(OH)$_2$D$_3$ or other deltanoids, but also demonstrating that several other types of neoplastic cells show

similar responses. These include colon cancer, breast cancer, prostate cancer, neuroblastoma, osteosarcoma, squamous cell carcinoma, and malignant melanoma. In addition, normally developing immature cells can be induced to differentiate by $1,25(OH)_2D_3$, for example keratinocytes, myoblasts, and perhaps hematopoietic cell precursors. Specific markers by which these phenotypes can be recognized are summarized, and sample references are provided, in Table I. It is apparent that $1,25(OH)_2D_3$ is a powerful differentiating agent that targets diverse cell types, but its physiological role in development remains to be fully determined. Although data from vitamin D receptor (VDR) knockout mice suggest that $1,25(OH)_2D_3$ can function in differentiation of normal cells (Chapter 20), most *in vitro* studies with normal immature cells used concentrations of $1,25(OH)_2D_3$ in great excess over the physiological levels. While it can be argued that concentrations of $1,25(OH)_2D_3$ in specific tissue niches in which precursor cells differentiate can be higher than the concentrations found in the plasma, a clearer assessment of the physiological role of $1,25(OH)_2D_3$ in normal differentiation may be achieved by studies that utilize low nanomolar or subnanomolar concentrations of $1,25(OH)_2D_3$.

B. Initial Signals for Differentiation Effects of $1,25(OH)_2D_3$

Cells that differentiate when exposed to $1,25(OH)_2D_3$ usually express VDR [20–23]. In several studies, evidence that VDR is strictly required for the $1,25-(OH)_2D_3$–induced differentiation was obtained. For instance, transfection of the VDR conferred differentiation responsiveness to $1,25(OH)_2D_3$ in WEHI-3B D+ murine myelomonocytic cells, which lack inducible VDR expression [24]. In VDR knockout mice, 12-O-tetradecanoylphorbol-13-acetate, but not $1,25(OH)_2D_3$ [25] or 19-nor-1,25-dihydroxyvitamin D_2 [26], induced differentiation of bone marrow-committed myeloid stem cells to monocytes/macrophages. This indicates the requirement of VDR for $1,25(OH)_2D_3$–induced monocyte/macrophage differentiation. Similarly, VDR is required for morphogenesis and negative growth regulation in the mammary gland [27].

VDR is a nuclear protein that may also shuttle to and from the cytoplasm [28] (reviewed in Chapter 22). Thus, liganded VDR, often acting as a heterodimer with one of the three members of the retinoid X receptor (RXR) family is the principal mediator of the effects of deltanoids, as described in detail in Chapter 13. There is, however, intriguing evidence that $1,25(OH)_2D_3$ and especially some other deltanoids have direct

cell membrane effects that modify, or enhance, the VDR-transmitted signals. This is extensively discussed in Chapter 23. Although the membrane effects originally described in the enterocytes are able to account for the very rapid calcium transport in the intestine ("transcaltachia" [29,30]), several reports suggest that the increased therapeutic ratios (i.e., the ratios of differentiation to calcemic potencies) of $1,25(OH)_2D_3$, and some low-calcemic deltanoids may be due, in part, to the auxiliary effects of non-VDR mediated actions of these compounds [31–33].

Nonetheless, it is most likely that the actions of VDR-RXR heterodimers that modulate gene expression transmit the differentiation signals to the basal transcription machinery by interacting with vitamin D response element (VDRE) sequences of the DNA molecule [34,35]. A large assortment of nuclear receptor co-activators, such as DRIP/Mediator and SRC/p160 [36–39] and co-repressors (e.g., SMRT and N-CoR) [38,40–42] has been identified, which provides positive or negative regulation of the VDR transcriptional activity. In addition to the "classic" VDRE sequences that transactivate vitamin D–responsive gene transcription, "negative" VDREs have also been characterized that inhibit transcription of certain genes, e.g., parathyroid hormone gene [43,44]. Interestingly, in myeloid leukemia cells, promyelocytic leukemia zinc finger (PLZF) protein and the chromosomal translocation products, such as promyelocytic leukemia-retinoic acid receptor alpha (PML/RARα) and PLZF/RARα fusion proteins also repress the differentiating action of VDR by binding and sequestering this receptor [45,46]. The regulatory effects of VDR co-modulators are discussed in detail in Chapters 14 and 16.

How the initial vitamin D–induced gene expression leads to the acquisition of a new functional phenotype, i.e. differentiation, is one of the current mysteries, as among the known direct target genes of VDR only one, p21Cip1, has a possible relevance to the differentiating actions of $1,25(OH)_2D_3$ in tissues other than bone [14,47]. Thus, with regard to differentiation of most cell types, the links from VDR-initiated events to the downstream targets of $1,25(OH)_2D_3$ still need to be found.

C. Pathways That Participate in $1,25(OH)_2D_3$ Signal Propagation

Pharmacologic inhibitors of several signaling pathways reduce, to varying extents, $1,25(OH)_2D_3$-induced differentiation in diverse model systems [13,48–54] encouraging the belief that these pathways participate in differentiation signaling.

TABLE I   Examples of Cellular Models of Differentiation Induced by 1,25(OH)$_2$D$_3$ and Deltanoids

Differentiation marker	Known function	Comments	References
Hematopoietic cells (HL60, U937, THP-1, UF-1, WEHI-3)			
CD14	LPS cell-surface binding protein	Early monocytic differentiation	[16,113]
CD11b	Cell surface protein (integrin αM)	General myeloid differentiation	[220,272,273]
Nonspecific esterase	Cytoplasmic hydrolytic enzyme	Monocytic differentiation	[16,256,274]
Superoxide anion	Component of oxidative burst	Phagocytic activity	[256,275–277]
Morphologic changes[a]			[102]
Colon cancer cells (Caco-2, primary adenoma and carcinoma lines)			
Alkaline phosphatase	Brush border-associated hydrolase	Intestinal and placental isozymes	[21,89,278–280]
Carcinoembryonic antigen (CEA)	Adhesion molecule	Early development protein	[281,282]
Osteoblast-like cells (MG-63, ROS 17/2.8, MC3T3-E1)			
Osteocalcin	Osteoblast-specific noncollagenous protein	Late osteoblastic differentiation	[283–285]
Alkaline phosphatase	Hydrolytic enzyme	Bone mineralization	[283,286,287]
Prostate cancer cells (LNCaP, PC-3)			
Prostate-specific antigen (PSA)	Serine protease	Secreted by prostate epithelial cells	[288–292]
Prostate-specific acid phosphatase	Protein tyrosine phosphatase	Prostate growth regulating enzyme	[291]
E-cadherin	Calcium-dependent cell adhesion molecule	Major epithelial cadherin	[290,293]
Breast cancer cells (MCF-7, T47D, MDA-MB-231, MDA-MB-436, BT-20, SK-BR-3, UISO-BCA-4)			
Intracellular lipid droplets	Storage/precursor material		[294–297]
Casein	Major milk protein		[295,297]
Neuroblastoma (LA-N-5)			
Acetylcholine esterase	Serine hydrolase	May regulate neurite outgrowth	[298–300]
Neurite outgrowth			[299,300]
Melanoma cells (B16)			
Tyrosinase	Copper-containing oxidase	Key enzyme in melanin synthesis	[301,302]
Squamous cell carcinoma (SCC13, SCC25)			
Keratin 1	Fibrous scleroprotein	Structural skin component	[303]
Transglutaminase	Calcium-dependent crosslinking enzyme	Keratinocyte-specific form	[304]
Involucrin	Glutamine-rich transglutaminase substrate	Cornified cell envelope constituent	[303,305,306]
Keratinocytes			
Transglutaminase	Calcium-dependent crosslinking enzyme	Keratinocyte-specific form	[304,307,308]
Involucrin	Glutamine-rich transglutaminase substrate	Cornified cell envelope constituent	[116,305,308]
Cystatin A	Cysteine proteinase inhibitor	Cornified cell envelope constituent	[309,310]
Cornified envelope formation			[307,311]
Muscle cells (C2C12)			
Myosin	Contractile protein	Late differentiation	[49,312]
Creatine kinase	ATP metabolizing enzyme		[49,313]

[a] Morphologic changes can be recognized in many forms of differentiation.

1. PROTEIN KINASE C

A number of early studies linked several isoforms of PKC to differentiation [55–59]. Following the observation by Martell, Simpson, and Taylor [60] that treatment of HL60 cells by 1,25(OH)$_2$D$_3$ increases cellular TPA receptors, which implies increased abundance of PKC, the Hannun Laboratory showed that 1,25(OH)$_2$D$_3$ increases the mRNA for isoforms α and β of PKC in

these cells [61]. These results were duplicated in many other laboratories, and PKC inhibitors or antisense oligonucleotides to this enzyme were demonstrated to interfere with $1,25(OH)_2D_3$-induced differentiation [49,51,62], further implying a role for at least some isoforms of PKC in differentiation induced by $1,25(OH)_2D_3$. If its role could be established, PKC would provide a central position in a logically pleasing sequence of events that led from an exposure of a cell to $1,25(OH)_2D_3$ towards differentiation. First, the lipid-soluble $1,25(OH)_2D_3$ may interact with cell membrane lipids or activate membrane-associated phospholipases [63] directly or through the still elusive membrane receptor, to generate a rise in phospholipid-derived intracellular calcium ($[Ca^{2+}]_i$). As the result of raised $[Ca^{2+}]_i$ and DAG concentrations, several PKC isoforms can be activated [64,65], and the signal can be propagated further by activating regulators of other signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway. Known examples of such links are the phosphorylation of Raf1 by PKCα in colon cancer CaCo-2 cells [66], and the translocation of MAPK ERK1/2 to the nucleus [67]. Another potential link is the regulation of VDR by PKC activation [68–70]. However, several major difficulties have so far precluded a full assessment of the role of PKC activity in differentiation. These include its presence in multiple isoforms with overlapping properties, and the fact that full inhibition of cellular PKC activity is usually incompatible with cell survival.

2. Phosphatidylinositol 3-Kinase

Effects of $1,25(OH)_2D_3$ originating in cell membranes are also linked to several other signaling pathways in still ill-defined ways. The phosphatidylinositol 3-kinase (PI3-K)/AKT signaling has been implicated in $1,25(OH)_2D_3$-induced differentiation of THP-1 human leukemia cells [48], HL60 cells [71–73], and keratinocytes, in which the membrane receptor for $1,25(OH)_2D_3$ was reported to be annexin II [54].

3. MAP Kinase Pathways

Although it is possible that MAPK activation also originates at the cell membrane, particularly in intestinal cells, osteoblasts, and chondrocytes, where the activation of MAPKs is very rapid [74], in other cell systems up-regulation of MAPKs can take place in a matter of hours and days, not only seconds or minutes, and genomic actions of $1,25(OH)_2D_3$ appear to be essential. In these model systems, which include HL60 leukemia cells, three MAPK pathways have been extensively studied. These pathways utilize ERKs, JNKs, and p38 MAPKs as the principal signal transmitters [75], as illustrated in Fig 1.
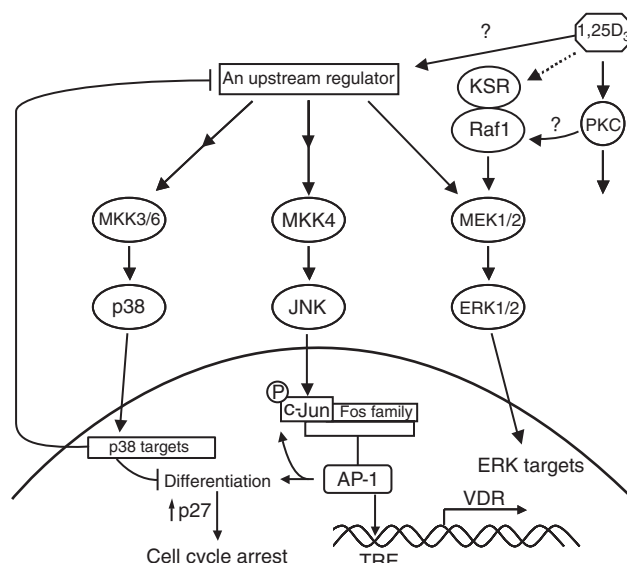
Figure 1   The MAP kinase pathways, which are up-regulated by $1,25(OH)_2D_3$ in leukemia cells. The ERK and JNK pathways have positive effects on differentiation [13,54], while the p38 MAPK pathway has an inhibitory effect on monocytic, but not granulocytic differentiation of HL60 cells [91,93]. Also shown is the potential role of the AP-1 transcription factor, which may act as an intermediary positive effector of $1,25(OH)_2D_3$ signals by upregulating the expression of VDR [98]. The dotted line depicts the upregulation by $1,25(OH)_2D_3$ of kinase suppressor of Ras (KSR) [86], which is presumably indirect.

The MEK/ERK MAPK module is activated by a sequence of kinase reactions that are initiated at the cell membrane by extracellular signals, which interact with receptors that include growth factor and cytokine receptors [76–79]. This cascade of phosphorylations leads to DNA replication, differentiation, and enhanced cell survival, the outcomes depending on the duration of the signal as well as the activity of other signaling pathways. The current paradigm is that these membrane events activate the small G protein Ras, which sequentially activates Raf1, MEK1, then ERK1/2, which translocates to the nucleus and phosphorylates transcription factors, thus increasing their activity [76–79]. Recently, however, another component, kinase suppressor of the Ras (KSR1), has been shown to be an intermediary between Ras, Raf, and MEK [50,80–82]. Although its function has been argued to be a kinase that phosphorylates and thus activates Raf1 [83,84], or a scaffold that brings Ras, Raf, and MEK together [85], it is clear that KSR facilitates the actions of the ERK MAPK pathway irrespective of its mode of action. Interestingly, KSR is up-regulated by $1,25(OH)_2D_3$ in HL60 cells, and appears to amplify the differentiation signal provided by nanomolar concentrations of $1,25(OH)_2D_3$ [50,86].

Activation of ERK2 by $1,25(OH)_2D_3$ has been found in many differentiation systems. For instance, "rapid

and transient" activation has been reported in NB4 acute promyelocytic leukemia cells [87], normal human keratinocytes [54,88], CaCo-2 colon cancer cells [89], and HL60 human myeloid leukemia cells [13,90]. While all these examples were found to be transient, the scale of "rapidity" varied from 30 sec [87], to several hours [13]. In the case of HL60 cells, Wang and Studzinski [13] found that the period of ERK2 activation following exposure to $1,25(OH)_2D_3$ corresponded to the time that these cells continued to proliferate before $1,25(OH)_2D_3$-induced cell cycle block became apparent. Similarly, Gniadecki [88] reported that in normal human keratinocytes $1,25(OH)_2D_3$ stimulated the activity of MAPKs and DNA synthesis. Together with the finding by Song *et al.* [87] of MAPK activation by $1,25(OH)_2D_3$ in NB4 leukemia cells in which $1,25-(OH)_2D_{3,}$ when administered alone, does not induce differentiation, these observations raise the question regarding the nature of the association between ERK activation and $1,25(OH)_2D_3$-induced differentiation. It is possible that ERK facilitates the early, proliferative phase of differentiation [9,11,12,91], thus increasing the numbers of differentiated cells, rather than being related to the expression of the differentiated phenotype. This is consistent with the observation that the MEK/ERK inhibitor PD98059 substantially reduces, but does not totally prevent, $1,25(OH)_2D_3$ induced differentiation [13], and the report that transformation of immortalized keratinocytes with Ha-RAS oncogene, an upstream regulator of the MEK/ERK pathway, results in resistance to the antiproliferative action of $1,25(OH)_2D_3$ [92].

The stress and pro-inflammatory cytokine-activated p38 MAPK has a complex relationship to $1,25(OH)_2D_3$-induced differentiation [75,93–96], as well as to apoptosis [97]. It has been reported that p38-mediated signals are necessary for induction of osteoclast differentiation, but not for osteoclast function [96], and that in keratinocytes $1,25(OH)_2D_3$ inhibits its activation [95]. In contrast, in HL60 cells $1,25(OH)_2D_3$ and other deltanoids activate p38, though inhibition of its activity by specific inhibitors SB202190 or SB203580 actually increases differentiation [93,94,98]. It was postulated that this paradoxical effect is due to the presence of a negative feedback mechanism that regulates several MAPK pathways (Fig. 1), and this explanation is consistent with the finding of increased JNK pathway activity, as well as enhanced differentiation, in HL60 cells treated with the p38 MAPK inhibitors SB202190 or SB203580 and $1,25(OH)_2D_3$ [93,94].

The JNK MAPK pathway activation by $1,25(OH)_2D_3$ in general has a positive effect on differentiation. In addition to HL60 cells [53,93,94], this pathway has been shown to be involved in stimulation of CaCo-2 cell differentiation [89], and, together with p38, in sensitization

of human breast cancer cells MCF-7 to $1,25(OH)_2D_3$–induced growth inhibition [99]. In contrast to ERK MAPK pathway activation which is transient, the JNK pathway activity increases more slowly after an exposure to $1,25(OH)_2D_3$, and in HL60 cells parallels the growth inhibitory effects of $1,25(OH)_2D_3$ [53]. Thus, in HL60 cells, enhanced JNK activity is a feature of late stages of monocytic differentiation, and perhaps is responsible for its maintenance.

4. THE CDK5/P35 PATHWAY

The pathways described above have been implicated in $1,25(OH)_2D_3$–induced differentiation by showing that their inhibition by pharmacological agents and dominant-negative or antisense constructs diminishes differentiation, suggesting that they are necessary for, or contributory to, signaling of differentiation. Whether the enhanced activity of any of these pathways is sufficient to induce the differentiated phenotype has not been unequivocally shown. One exception is the demonstration that transfection of a cyclin-dependent kinase 5 (Cdk5)-expressing plasmid can lead to the expression of markers of early monocytic differentiation (CD14 and NSE) if a cyclin-like protein p35Nck5a is present [100]. Since the expression of both Cdk5 and p35 is upregulated by $1,25(OH)_2D_3$ [100–102], this provides a mechanism by which $1,25(OH)_2D_3$ can signal the early stages of monocytic differentiation. A confirmation of this role of the Cdk5/p35 module was obtained by finding that monocytes in p35 knockout mice were deficient in NSE, a monocyte-specific esterase used as a cell-specific marker [103]. The role of the ERK pathways as the upstream regulator of Cdk5/p35 was studied by Harada *et al.* [104] in differentiating neurons. The authors reported that the nerve-growth factor-induced differentiation in these cells was associated with a strong, sustained expression of p35 through activation of the ERK pathway, and that constitutive activation of ERK was necessary and sufficient for p35 induction. Furthermore, the mechanism by which activation of ERK induces expression of p35 involves the transcription factor Egr1, which was in these experiments induced by nerve-growth factor through the ERK pathway and appeared to mediate the induction of p35 by ERK [104]. Thus, while it is likely that MAPK pathways are upstream of Cdk5/p35, the evidence is currently not available for deltanoid–induced differentiation.

D. Role of General Transcription Factors in $1,25(OH)_2D_3$-Induced Differentiation

In addition to directly activating VDR to heterodimerize with a member of RXR family, which results in

transcription of VDRE-containing genes, several ubiquitous transcription factors appear to be involved, perhaps in a contributory way, in 1,25(OH)$_2$D$_3$–induced differentiation. These may act by interacting with the adjacent VDREs [105], by up-regulating VDR expression through its promoter region (e.g., ref. [99]), and in other ways, many of which remain to be elucidated.

1. ACTIVATOR PROTEIN-1 (AP-1)

ERK and JNK pathways activate members of the Fos and Jun families, as well as some related proteins, which dimerize in various combinations to form the AP-1 transcription factor [106,107]. Thus, AP-1 can integrate and transmit signals transduced by the MAPK pathways previously discussed. Early studies have shown, for instance, that the expression of c-Jun is increased during the 1,25(OH)$_2$D$_3$–induced differentiation of human myeloid cells [53,108], and coordinate occupancy of AP-1 sites and VDRE elements by their cognate transcription factors provide a possible model for the reciprocal relationships between different cellular phenotypes and functional activities such as those that occur during differentiation [109,110]. For example, a composite AP-1 steroid hormone element that responds to 1,25(OH)$_2$D$_3$ mediates differentiation-specific gene expression of human keratin-1 [111]. There is also evidence for functional cooperation between VDR and Ras-activated Ets transcription factors in 1,25(OH)$_2$D$_3$–mediated induction of CYP24 gene expression [112]. Liu and Freedman [105] conducted an extensive study of such transcriptional synergism between VDR and nonreceptor transcription factors, and concluded that its functional basis appears to be at the level of cooperative DNA binding.

AP-1 activation by 1,25(OH)$_2$D$_3$ has been described in diverse differentiation systems. These include HL60 cells [113], colon cancer CaCo-2 cells [89], osteoblastic cells [114], keratinocytes [115,116], and breast cancer cells [99]. Interestingly, the pathways that signal AP-1 activation are apparently cell type-specific. For instance, the p38 and JNK MAPK pathways cooperate to activate VDR by c-Jun/AP-1 in breast cancer cells [99], while in keratinocytes and HL60 leukemia cells AP-1 activation is attributed to both the ERK and JNK pathways [54,93]. Thus, AP-1 transcription factor appears to be an important integrator of converging differentiation pathways.

2. SP1 TRANSCRIPTION FACTOR

The Spl, a 95–105 Kd protein, is ubiquitously expressed in growing cells, and, usually in combination with other factors, acts as a transcriptional activator of many housekeeping genes [117–121]. Its role in 1,25(OH)$_2$D$_3$–induced differentiation has been suggested

in human myeloid leukemia cells. Specifically, the DNA binding of Spl was found to be increased following an exposure of HL60 cells to 1,25(OH)$_2$D$_3$ [10,113,122], while in U937 cells Spl may participate in the 1,25(OH)$_2$D$_3$–induced expression of the CD14 monocyte marker, which has several Spl sites in its promoter [123]. It was also shown that up-regulation of p27Kipl in 1,25(OH)$_2$D$_3$-treated U937 cells can be mediated by Spl [124], though more recent work indicates that forkhead transcription factors play a major role in the transcriptional regulation of p27Kip1 expression [125,126].

3. OTHER TRANSCRIPTION FACTORS

Undoubtedly, many other transcription factors contribute to 1,25(OH)$_2$D$_3$-induced differentiation and the eventual cell cycle arrest and some of these, notably c-Myc, will be discussed relative to cell cycle control. The important distinction between factors, which regulate the expression of new genetic programs, and factors which carry out functions of the differentiated cells is not easy to make at present.

III. CELL CYCLE CONSEQUENCES OF DELTANOID-INDUCED DIFFERENTIATION

A. General Features of Cell Cycle Machinery

1. CELL CYCLE COMPARTMENTS AND CHECKPOINTS

The consecutive progression through four distinct phases of the cell cycle called G1, S, G2, and M results in proliferation of eukaryotic cells (Fig. 2). DNA replication occurs during the S phase; chromosome separation (karyokinesis) takes place during the M phase, and is followed by cell division (cytokinesis); G1 and G2 are gap or growth phases. The G1 phase can be further subdivided into early G1, or post-mitotic G1, mid-G1, in which principal cell growth takes place, and late-G1, in which final preparations for DNA replication occur. The G2 phase is thought to be necessary for monitoring of chromosome replication and preparations for mitotic spindle assembly [127–129]. Cells that are not actively dividing may either be permanently removed from these cycling phases by terminal differentiation, senescence or apoptosis, or be temporarily arrested in a noncycling quiescent state known as G0 if the cells have the G1 DNA content, though quiescence can also occasionally take place in the G2 phase (G2 arrest). As mentioned above, specific nuances have been described, but the remarkable feature of the cell cycle is the conservation, from yeast to
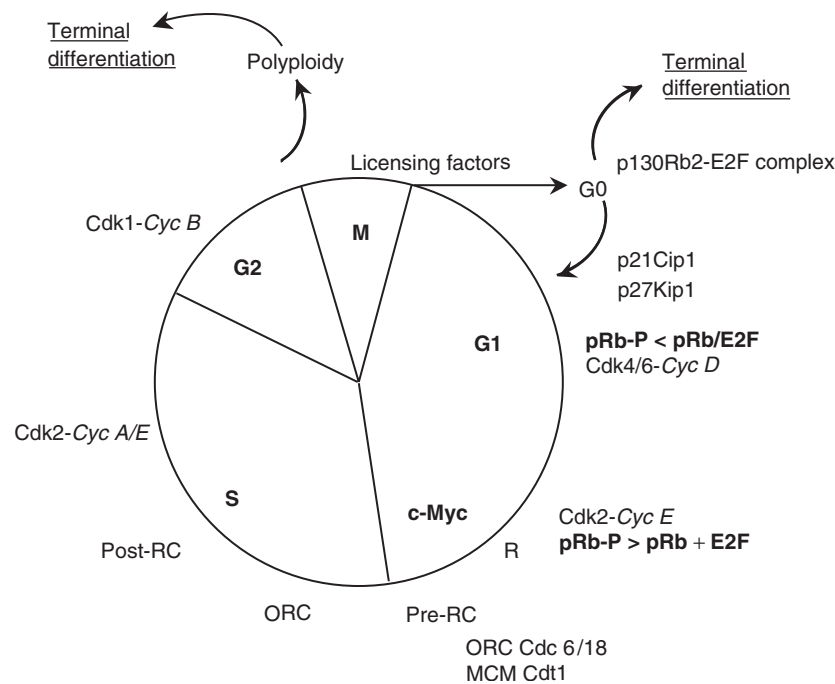
FIGURE 2 The general concept of the cell cycle, and examples of factors that control DNA replication and the cell cycle traverse. The principal locus for terminal differentiation is in G0, but can occur in G2, with polyploidy. In early G1 the level of phosphorylated retinoblastoma protein (P-pRb) is less (<) than the level of hypophosphorylated pRb protein complexed with transcription factor E2F (pRb/E2F), while in late G1 the level of P-pRb is high, and it is not complexed with E2F. This and the increasing Cdk2/Cyclin E activity allow passage through the Restriction point (R). Additional details of the controls of cell cycle progression are provided in the text and illustrated in Figs. 3–6. RC, replicative complex; ORC, origin recognition complex; MCM, mini-chromosome maintenance complex; licensing factors, ORC, MCM, and other components of the pre-RC complex; Cyc, cyclin.

mammalian cells, of the basic regulatory mechanisms and components.

A series of regulatory steps, referred to as *checkpoints*, control the traverse of the various compartments of the cell cycle [130]. A simple definition of a checkpoint is that it is a mechanism that prevents progression to the next part of the cell cycle unless and until the preceding part has been satisfactorily completed. Such checkpoints operate in each phase of the cell cycle, and although the precise mechanisms are in most cases unclear, DNA damage is known to activate two protein kinases, Chk1 and Chk2, which then mediate cell cycle arrest [131,132]. In addition, several regulators downstream from Chk1 or Chk2, such as p53, have been shown to have importance in checkpoint control. Interestingly, these regulators of cell cycle progression may also influence cellular decisions to differentiate [133].

The forerunner of checkpoints was described as the restriction (R) point in mid to late G1 phase. Based on work in his own and in other laboratories, Pardee defined a transition in G1 phase which commits a cell to initiate DNA replication [127]. Additional work indicated that phosphorylation of the retinoblastoma susceptibility protein (pRb) may provide the principal mechanism for the transition through the R point [134]. Subsequent passage through the S phase can also be controlled by the S phase checkpoints [135].

The final result of the cell cycle traverse is a faithful replication and accurate partitioning of genetic information. The fidelity of this partitioning is maintained by the G2 and the M phase checkpoints. The G2 phase checkpoints monitor the integrity of DNA and the accuracy of DNA replication, and the M phase checkpoints ensure correct chromosomal segregation and alignment. Each of these checkpoints arrests cell cycle progress to allow editing and repair of genetic information. Overall, the checkpoints assure that each daughter cell receives a full complement of genetic information intact and identical to the parental cell.

2. Mechanisms That Drive Cell Cycle Progression

Passage through the restriction points is propelled by the activity of a group of enzymes known as *cyclin-dependent kinases* (Cdks). Cdks are usually present throughout the cell cycle, and work in concert with cyclins, which are nuclear proteins whose levels oscillate in a cell cycle-dependent manner [136,137].

In general, there are at least nine levels at which the activity of Cdks can be controlled, as detailed in Fig. 3. The primary regulator of Cdks activity is cyclin binding, because Cdks and cyclins need to form a complex prior to activation. Since the protein levels of the cyclins dramatically change during the cell cycle, the binding of Cdks and cyclins is related to cyclin availability. The abundance of cyclins, like all proteins, is dependent on the balance between their synthesis and their degradation, the latter occurring by ubiquitin-dependent proteolysis [138].

The activity of the cyclin-Cdk complexes also depends on both activating and inhibitory phosphorylations. A known kinase, which can phosphorylate Cdks is cyclin activating kinase (CAK). CAK is comprised of cyclin H (regulatory subunit), Cdk7 (catalytic subunit), and MAT1 (assembly factor) [139,140]. This complex phosphorylates threonines (Thr) 161/160 on Cdk1 (formerly Cdc2) and Cdk2, respectively [141]. Phosphorylation of this site is necessary for the cyclin-Cdk complex to be activated. Cdk inhibitory phosphorylation sites include Thr14 and Tyr15. Regulation of these sites is achieved by a group of proteins that include Cdc25A, B, C (phosphatases), as well as wee1 and mik1 (kinases). The phosphatases activate the cell cycle by cleaving the phosphate groups on Thr14 and Tyr15 residues of the Cdk, while the kinases are inhibitory by phosphorylating the same sites. Cdc25A is involved in the G1/S checkpoint [142], while Cdc25B and C, and human wee1-like kinase, regulate the traverse through the G2/M phases [143–145]. If DNA damage occurs prior to or in the S phase, Chk2 phosphorylates Cdc25A and targets it for degradation, while G2/M transition is regulated by Chk1 and Chk2 by phosphorylation of Cdc25C at Ser216 [146].

Another level of Cdk regulation is provided by the Cdk inhibitory proteins (CDKIs), which prevent Cdk activation, generally by binding to the kinases, thus preventing their activation by cyclins, as described below.
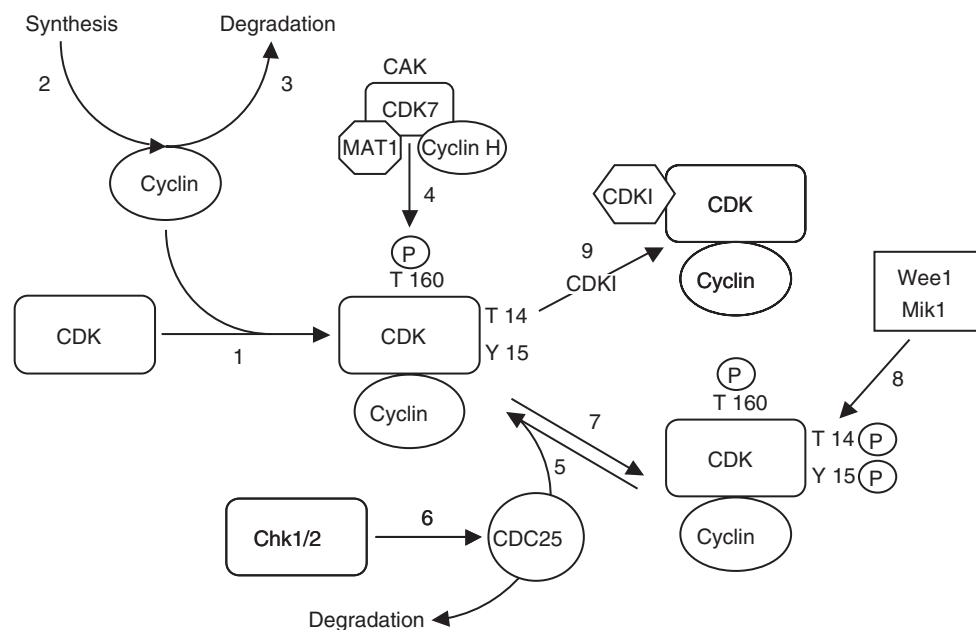
FIGURE 3   The central paradigm for the control of the cycle traverse by cyclin-dependent kinases (Cdk). Cdk activity is primarily activated by cyclin binding, phosphorylation of Thr160 by cyclin activating kinase (CAK), and dephosphorylation of Thr14 and Tyr15 by Cdc25 phosphatases. Phosphorylation of Thr14 and Tyr15 by wee1 and mik1 kinases as well as binding of Cdk inhibitors (CDKIs) result in Cdk inhibition. In this figure, the centrally placed canonical Cdk/cyclin complex is the driving force for cell cycle progression.

B. Regulation of Cell Cycle Progression

1. THE G1 TO S PHASE TRANSITION

While the cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes control the entry into the S phase, these complexes are in turn controlled by families of G1/S regulatory polypeptides, the CDKIs [136] (Fig. 4). Specifically, cyclin D, E, and A-dependent kinases are negatively regulated by a family of CDKIs that consists of p21Cip1, p27Kip1, and p57Kip2. Although all three of these inhibitors block progression through the G1 phase, each is usually activated by different stimuli. The expression of p21Cip1 can be under the transcriptional control of the p53 tumor suppressor gene, activated by DNA damage [147], but may also be independent of p53 [148]. The increase in p21Cip1 protein levels may lead to the inhibition of cyclin D-Cdk4/6 activity, which contribute to the G1 arrest. A second mechanism of action of p21Cip1 may be related to its ability to bind to the proliferating cell nuclear antigen (PCNA), a molecule involved in DNA replication and repair [149].

Like p21Cip1, p27Kip1 inhibits the activity of the G1/S cyclin Cdk complexes. For instance p27Kip1 participates in G1 arrest produced by the exposure of fibroblasts derived from mink lung to the transforming growth factor β (TGFβ), and by cell-cell contact [150]. In actively dividing cells, p27Kip1 is phosphorylated by cyclin E-Cdk2 complex in the nucleus [151], and its abundance regulated by p45Skp2, also known as SCF/Skp2, which promotes ubiquitin-mediated degradation of p27Kip1 [152,153]. However, cytoplasmic control is also suggested by another, not yet identified ubiquitin ligase, that is present in the cytoplasm and is Thr187 phosphorylation-independent [154,155]. As mentioned above, the forkhead family of transcription factors can also up-regulate expression of p27Kip1 [125,126]. The inducers of p57Kip2 expression are unknown.

Another family of regulatory peptides are the INK4 proteins, which include p16(INK4A), p15(INK4B), p18(INK4C), and p19(INK4D). These proteins specifically block cyclin D-Cdk 4/6 activity, leading to a G1 phase arrest [156]. The INK4 proteins inhibit Cdk4
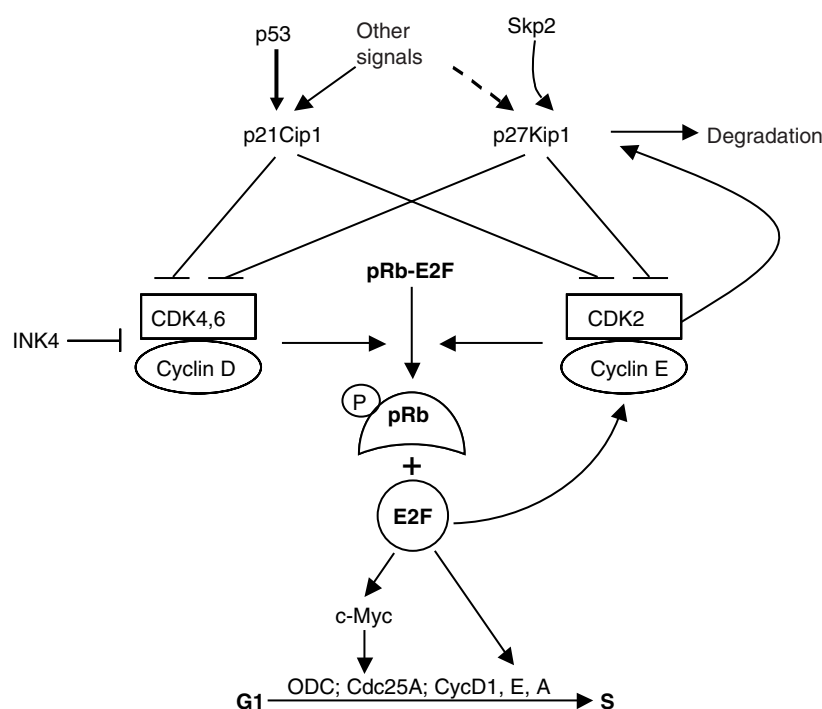
FIGURE 4 Control of G1 to S phase transition by the pRb-E2F pathway. Phosphorylation of pRb by active Cdks releases E2F transcription factors, which activate, directly or indirectly, genes whose products are required for DNA replication [159,161]. The activity of Cdks can be controlled by factors shown in Fig. 3, and these include CDKIs belonging to the INK4 and Cip/Kip families. ODC, ornithine decarboxylase; Cyc, cyclin. Only a few examples of over 600 known c-Myc target genes are shown here; this growing list can be accessed at www.myc-cancergene.org.

and 6 by preventing the binding of cyclin D, but also inhibit the activation of the formed Cdk 4/6-cyclin D complexes. Treatment of human keratinocytes with TGF-β results in an increase in p15(INK4B) expression and its association with Cdk4 and 6 [157]. While there is a large body of evidence showing the regulation of Cip/Kip CDKIs by 1,25(OH)$_2$D$_3$ and its analogs (see below), the control of INK proteins by deltanoids has not been established.

An important event for the G1-S transition appears to be the phosphorylation of the tumor suppressor, pRb, as shown in Fig. 4 [158,159]. pRb, and other pRb-like "pocket" proteins (p130/Rb2,p107), are believed to control the entry into the S phase by interacting with a member of the E2F transcription factor family. This family is composed of at least five proteins (E2F 1–5), which are active when they form heterodimeric complexes with one of the E2F-related transcription factors, DP-1, 2, or 3. In its simplest form, the current hypothesis is that hypophosphorylated pRb binds to E2F, preventing cell entry into the S phase. Upon increased level of phosphorylation, pRb frees E2F, which in its heterodimeric complex with DP is capable of activating genes necessary for S phase initiation in part mediated by c-Myc/Max transcription factor [160–164]. However, the actual situation appears to be more complicated. Recent reports [165,166] suggest that gene repression by pRb also involves modulation of chromatin architecture. The proposed mechanism rests on the finding that histone deacetylase HDAC1 physically interacts with pRb through the "pocket" domain and recruits HDAC1 to E2F. The complex of these three proteins binds to E2F target promoters. HDAC1 may then facilitate the removal of highly charged acetyl groups from core histones, leading to a tight association between the nucleosomes, which prevents the access of transcription factors to their cognate elements in the gene promoters [156].

Mitogenic signals (e.g., growth factors, serum compounds) that stimulate cell progress through G1 coincide with increased expression of D cyclins. This extracellular regulation of cyclin D isoforms is not observed with other cyclin proteins. The current classical scenario is that cyclin D then forms a complex with Cdk4 and/or Cdk6, and its activity is regulated by the mechanisms described above. The activated cyclin D-Cdk 4/6 complexes then phosphorylate pRb and release E2F, or relieve the chromatin configuration constraints described above, leading to G1 progression.

The cyclin D complex alone does not dictate control of progression through G1. Cyclin E is another G1 cyclin, which is synthesized later in the cell cycle than cyclin D, peaking at the late G1/S phase boundary. The expression of cyclin E is mitogen-independent, and

cyclin E forms an active complex with Cdk2. One level of regulation for the cyclin E-Cdk2 complex is through protein phosphatase Cdc25A, which cleaves the phosphate groups on the Thr14 and Tyr15 residues of Cdk2, and activates the Cdk2-cyclin E complex [167]. Cdc25A activity may in turn be regulated by phosphorylation by Chk1 [168]. c-Myc has been shown to regulate the expression and phosphorylation, and therefore the activity, of Cdc25A [169]. Cyclin D-Cdk4 and cyclin D-Cdk6 complexes are believed to trigger pRb phosphorylation, but cyclin E-Cdk2 complex also contributes to the phosphorylation of pRb in late G1, leading to cell entry into the S phase, where pRb phosphorylation is maintained by cyclin E/Cdk2, or cyclin A/Cdk2.

2. CONTROL OF DNA REPLICATION LICENSING

The S phase is the period where DNA replication takes place, and the basic machinery for this process is well conserved from yeast to mammals [170]. It is permitted by the rising level of Cdk activity, and is initiated on many sites on chromosomes, designated as "replication origins", which can exist in two states [171]. In G1 phase, a multiprotein complex, the prereplicative complex (pre-RC), is assembled, but once DNA replication is initiated, the complex has fewer components ("post-RC"), which persists to the end of mitosis, and does not permit re-replication of DNA. At that time point proteolytic activity destroys the cyclins and other nuclear proteins, and Cdk activity becomes low. These two states of replication origins, separated by Cdk activity, generally ensure that the S phase and mitosis alternate.

When chromatin becomes competent for DNA replication by the presence of pre-RC on the replication origin, it is considered to be "licensed." Components of the pre-RC, the "initiator" proteins, include the Origin Recognition Complex (ORC), Cdc6/18, Cdt1 and Mini-Chromosome Maintenance (MCM) proteins. Although the DNA consensus sequences have not been found in organisms other than yeast, ORC proteins have been found in several eukaryotes, and all have ATP binding sites, consistent with the requirement of ATP for the initiation of DNA replication [172]. Cdc6/18 mammalian homologs may be related to M checkpoint control [173], while Cdt1 (Cdc10-dependent transcript 1), which also has peak protein levels at G1/S boundary [174], is associated with DNA replication checkpoint control. The six MCM proteins form a hexameric complex, approximately 600 kD in size, which may function as a replicative helicase [175]. Importantly, Cdt1 binds tightly to a DNA replication initiation inhibitor (Geminin) and this inhibits MCM loading. Licensing in G1 phase is permitted after the end of mitosis, when

Geminin is destroyed by the Anaphase Promoting Complex (APC) – ubiquitin system [176].

Thus, the current model posits that ORC associates with the replication origins throughout the cell cycle, and when the cells exit mitosis Cdc6/18 and Cdt1 are loaded on chromatin, and in turn aid loading of MCM on the pre-RC complex, thus completing licensing [174]. The licensed complex can now be activated for DNA replication by a protein kinase, such as cyclin E/Cdk2 or Dbf4-dependent kinase (DDK) [177], and the DNA replicating machinery (e.g., Cdc45, replication protein A (RPA), DNA polymerase α and ε) is recruited to the initiation sites [178–181]. To further facilitate replication, a SCF-ubiquitination complex, which destroys Cdk inhibitors, can be recruited to the pre-RC by a cyclin-binding site on Cdc6/18 [182].

3. THE G2 AND M PHASE TRANSITIONS

Once the cell has faithfully replicated its genome, the next cellular function is to segregate this DNA into equivalent, or nearly equivalent, daughter cells. The central regulation for the transition from G2 to mitosis is by the cyclin B-Cdk1 complex, initially called *maturation (mitosis) promoting factor*, or MPF [183]. In general, the activity of this complex is governed by factors similar to those responsible for the G1-S transition, including Cdk-cyclin association and activating phosphorylation by CAK (Fig. 5). The CDKIs were not known to play a major role in the control of the G2/M traverse, but studies in S. Reed's laboratory indicate that the situation is more complex than previously believed [184,185].

Regulation of the Cdk1-cyclin B complex does include the G2/M specific phosphatase/kinase cell cycle regulatory proteins Cdc25C and perhaps Wee1-like tyrosine kinase. Cdc25C is a protein phosphatase, which cleaves the inhibitory phosphate groups at both Tyr15 and Thr14 on Cdk1 [186,187]. Cdc25C itself requires phosphorylation to be activated, and recent data support that Cdc25C is phosphorylated and activated by the cyclin B-Cdk1 complex, thus forming a positive feedback loop [188]. On the other hand, Wee1 and Mik1 phosphorylate these same sites, and thus act as inhibitor of the progression into mitosis [189].

In addition to the accurate duplication of the genetic material, cell cycle controls ensure its correct segregation into the daughter cells. This occurs at two levels: regulation of proteins that bind together the two chromatids, and control of centrosome duplication and spindle assembly.

The sister chromatids adhere to one another by the adhesive properties of multi-submit complex composed of several proteins, so far best characterized in yeast, collectively called *cohesin* [190]. Cohesin is
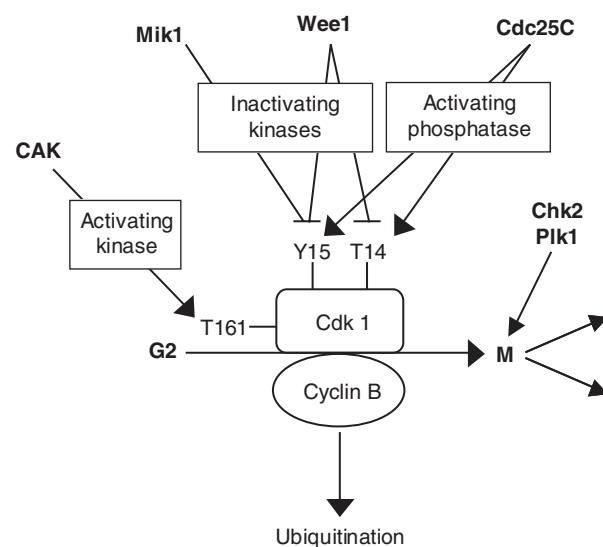
FIGURE 5 Control of G2 to M phase transition and the completion of mitosis. Cyclin B-Cdk1 complex is a central regulator of the transition from G2 to M. The activity of this complex is governed by factors similar to those responsible for the G1-S transition (see Fig. 4), including Cdk-cyclin association, activating phosphorylation of Thr161 as well as phosphorylation/dephosphorylation of Thr14 and Tyr15. See text for additional details. CAK, cyclin activating kinase.

destroyed by proteolytic cleavage of one of its subunits, Scc1/Med1, by a calcium-activated cysteine protease, related to caspases, known as "separase" [191]. In animal cells the dissolution of cohesion between the chromatids occurs in two steps, one at prophase, the other at anaphase, and only the latter requires separase [192]. Interestingly, separase is subjected to multiple levels of regulation. These include its phosphorylation by cyclin B-Cdk1 [193], and the inhibition of separase catalytic activity by securin [194]. Since both cyclin B and securin are ubiquitinated by APC and destroyed at the end of mitosis, this ensures orderly and precisely timed separation of the chromosomes at telophase.

Polo-like kinase (Plk-1) also regulates chromosome adhesion and other aspects of mitosis, including centrosome maturation and orientation [195]. The recent finding of co-localization of Plk1 and Chk2 suggests that there is a lateral communication between the mitotic checkpoint and the DNA integrity checkpoint [196].

The changes in cell cycle traverse and DNA replication that occur in numerous forms of differentiation have been previously reviewed [8]. Changes that specifically follow exposure to vitamin D derivatives have been less extensively studied, and with this background these will now be described.

C. Modulation of Cell Cycle Events by Deltanoids

The inhibition of cell cycle traverse by $1,25(OH)_2D_3$ and analogs has been investigated in normal and malignant keratinocytes [197–200], and in many other types of tumor cells, with myeloid leukemia providing an excellent *in vitro* model system for this purpose [26,201].

1. The G1/S Block

Deltanoids inhibit proliferation of diverse types of mammalian cells by arresting them in the G1/G0 phase of the cell cycle. While the exact sequence of events from VDR activation to G1/G0 arrest remains to be elucidated, and may not be exactly the same in all cell types, several pathways and cell cycle arrest effectors are already known to be involved. These are the up-regulation of protein levels of the Cdk inhibitors p21Cip1 and/or p27Kip1, upregulation of the retinoblastoma gene expression and reduced phosphorylation of pRb protein, and the inhibition of c-Myc expression [75,91,202–204].

a. Upregulation of p21Cip1 and p27Kip1   Elevated protein levels of the Cip/Kip family of CDKIs result

FIGURE 6   Inhibitory effects of $1,25(OH)_2D_3$ on the G1 traverse, which result in G1/S block. The key events in leukemia cells include: (1) An early up-regulation of RB gene expression, which sequesters E2F necessary for the expression of cyclin E; (2) Inhibition of p45Skp2 expression, which, together with reduced cyclin E levels and consequent reduced phosphorylation of p27Kip1, led to increased levels p27Kip1; (3) Progressively increasing sequestration of E2F resulting in further increase in p27Kip1 and decreases in c-Myc and other proteins required for DNA re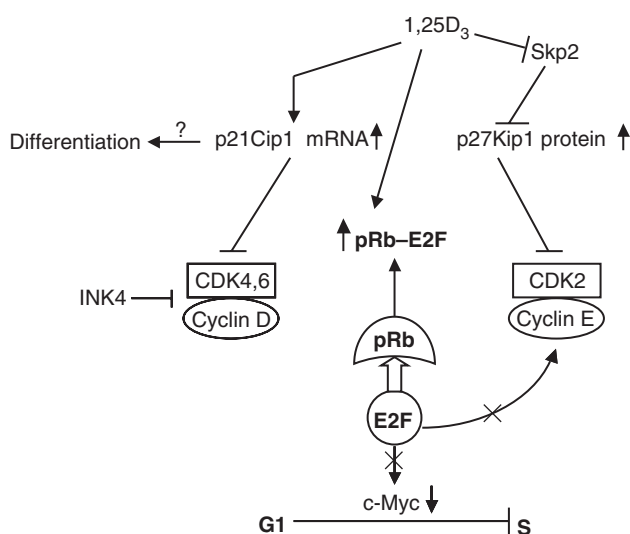plication. The direct activation of p21Cip1 gene may have an effect on other pathways that lead to differentiation, or contribute to the effects of $1,25(OH)_2D_3$ on the pRb/E2F pathway.

from the exposure to $1,25(OH)_2D_3$ and other deltanoids in many cell types (Table II), and may be a near universal feature of the antiproliferative effects of these compounds. In view of the importance of Cdk activity as the driving force for cell cycle progression, it is not difficult to understand that increased levels of CDKIs can target Cdk2 complexed to cyclin D, E, or A, and grind the cell cycle traverse to a halt. However, the mechanisms of the CDKI up-regulation are not entirely clear, and there are subtle differences between the antiproliferative effects of p21Cip1 and p27Kip1, while p57Kip2 is not known to have a role in antiproliferative effects of deltanoids.

Considerable excitement was generated when p21Cip1 was found to be up-regulated in a number of differentiation systems, including HL60 cells treated with $1,25(OH)_2D_3$ [148,205]. It was suggested that p21Cip1, and/or p27Kip1, not only promote the G1 arrest but also contribute to differentiation [47,206]. It seems, however, that while these Cdk inhibitors may not be solely responsible for the G1 block, the data regarding their role in differentiation are conflicting. For instance, mice lacking p21Cip1 undergo normal development [207], even though p21Cip1$^{-/-}$ embryonic fibroblasts show impaired arrest in G1 in response to DNA damage. An imbalance between growth and differentiation can be demonstrated in these cells, and in other *in vitro* cell differentiation systems with p21Cip1 knockouts. Keratinocytes which are p21/Cip1$^{-/-}$, and to a lesser extent those with p27Kip1 knockouts, have an increased proliferative potential [208]. With regard to differentiation, however, Harvat [209] showed that growth arrest resulting from overexpression of p21Cip1 in mouse primary keratinocytes is not sufficient to induce the expression of markers of differentiation. Further, in malignant counterparts of these cells, the squamous cell carcinoma (SCC) cells, not even growth arrest is clearly linked to p21Cip1, as $1,25(OH)_2D_3$ inhibited growth but reduced p21Cip1 levels *in vitro* and in SCC tumors [210].

In another system, the myelomonocytic cell line U937, Freedman's group noted transcriptional activation of the p21Cip1 gene by $1,25(OH)_2D_3$, and suggested that this is linked to differentiation of these leukemia cells [47]. Importantly, they identified a functional vitamin D response element (VDRE) in the promoter of the p21Cip1 gene, and noted that the p21Cip1 transcript can be detected as early as 2 h after $1,25(OH)_2D_3$ addition, consistent with p21Cip1 being a direct mediator of $1,25(OH)_2D_3$ action. However, in this system the up-regulation of p21Cip1 after exposure to $1,25(OH)_2D_3$ is transient and accompanied by a proliferative burst [14], which does not correlate with the onset of the G1 block that is observed 24–48 h

TABLE II   Examples of Up-Regulation of CDKI Levels by 1,25(OH)$_2$D$_3$ and Deltanoids

CDKI	Cell type	Functional effect/comment	References
Hematopoietic cells			
p21Cip1	HL60	Immediate early gene induced during monocytic differentiation/G1 arrest	[148,205,314,315]
p21Cip1	U937	Transcriptional activation of p21/induces monocytic differentiation	[47]
p21Cip1	U937	Cytoplasmic localization/anti-apoptotic effect	[316]
p21Cip1	U937	Antisense to p21 decreases differentiation	[317]
p27Kip1	HL60	Proliferation block/G1 arrest	[91,204,318,319]
p27Kip1	HL60	Antisense to p27 reverses G1 arrest	[201]
p27Kip1	U937	Proliferation block	[319]
p27Kip1	U937	Vitamin D receptor-independent upregulation of p27 gene	[124]
p21/p27	HL60	G1 arrest/little change in CDK4 activity	[320]
p21/p27	U937	Early proliferative burst followed by growth arrest and differentiation	[14]
p21/p27	UF-1	Granulocytic differentiation/proliferation block/G1 arrest	[220]
Prostate cancer cells			
p21Cip1	LNCaP	Proliferation block/G1 arrest	[211–213]
p21Cip1	ALVA-31	Proliferation block/G1 arrest	[213]
p21Cip1	PC-3	Proliferation block/differentiation	[290]
p21Cip1	DU-145	Proliferation block	[290]
p27Kip1	LNCaP	Increased association of p27 with Cdk2/Stabilization of p27/G1 arrest	[321,322]
p21/p27	LNCaP	Proliferation block/differentiation	[290]
Breast cancer cells			
p21Cip1	MCF-7	Proliferation block/G1 arrest/apoptosis	[323,324]
p21Cip1	MCF-7E	Proliferation block/G1 arrest	[214]
p21Cip1	MCF7/LCC2	Proliferation block/G1 arrest/transient upregulation of com1	[325]
p27Kip1	MCF-7	Proliferation block/G1 arrest	[326]
p27Kip1	SK-BR-3	Proliferation block	[326]
p21/p27	MCF-7	Proliferation block/G1 arrest/downregulation of c-Myc	[215,216,296,327,328]
p21/p27	BT20	Proliferation block/G1 arrest	[214]
p21/p27	ZR75	Proliferation block/G1 arrest	[214]
p21/p27	SUM-159PT	Proliferation block/Apoptosis	[329]
Pancreatic cancer cells			
p21/p27	BxPC-3	Early transient CDKI upregulation/proliferation block/G1 arrest	[218]
p21/p27	Hs 700T	Early transient CDKI upregulation/proliferation block/G1 arrest	[218]
p21/p27	SUP-1	Early transient CDKI upregulation/proliferation block/G1 arrest	[218]
Insulinoma cells			
p21Cip1	Beta TC(3)	G1 arrest/apoptosis	[330]
Colon cancer cells			
p21Cip1	HT-29	Proliferation block/G1 arrest/apoptosis	[331]
p21/p27	Caco-2	Co-association between Cdk2, p27Kip1 and cyclin E/proliferation block	[332–334]

(Continued)

TABLE II   Examples of Up-Regulation of CDKI Levels by $1,25(OH)_2D_3$ and Deltanoids—**Cont'd**

CDKI	Cell type	Functional effect/comment	References
Neuroblastoma cells			
p21Cip1	SH-SY5Y	Downregulation of Myc and Id2/induction of RARβ/proliferation block	[335,336]
p21Cip1	NB69	Downregulation of Myc and Id2/induction of RARβ/proliferation block	[335,336]
p21Cip1	SK-N-AS	Downregulation of Myc/induction of RARβ/proliferation block	[335,336]
p21Cip1	IMR5	Downregulation of Myc and Id2/induction of RARβ/proliferation block	[335,336]
p21Cip1	CHP134	Downregulation of Id2/induction of RARβ/proliferation block	[335,336]
p21Cip1	NGP	Downregulation of Myc and Id2/induction of RARβ/proliferation block	[335,336]

later, and is accompanied by markedly increased levels of p27Kip1 [47,91,204]. Thus, although p21Cip1 may initiate a cascade of unknown events that lead to the expression of the differentiated monocytic phenotype, it is unlikely to be directly responsible for the G1 arrest in leukemia, or SCC, cells. Such function, however, has been attributed to p21Cip1 in other cells, including prostate cancer [211–213], breast cancer [214–216], and parathyroid cells [217]. The role of binding of p21Cip1 to PCNA [149], the processive factor for DNA replication, remains to be elucidated.

The first demonstration that up-regulation of 27Kip1 is associated with the G1 arrest which takes place following $1,25(OH)_2D_3$–induced differentiation was reported by Wang *et al*. in 1996 [91]. They showed a sustained increase in p27Kip1 protein abundance that coincided with the appearance of the $1,25(OH)_2D_3$–induced G1 block in HL60 cells, and correlated with reduced kinase activity of Cdk6 and Cdk2 [91,204]. Further, reductions of the levels of p27Kip1 by several independent approaches reversed the G1 block, but not the differentiated phenotype [201]. Accordingly, these data clearly show that, at least in HL60 cells, p27Kip controls the $1,25(OH)_2D_3$–induced G1 block, but not the differentiated phenotype.

Similar findings have been obtained in several other systems, although the data cannot always be so clearly interpreted. For instance, the up-regulation of p27Kip1 is often accompanied by an up-regulation of p21Cip1 [214,218,219]. However, even in situations where p21Cip1 and p27Kip1 are both up-regulated by $1,25(OH)_2D_3$ or other deltanoids, increased levels of p27Kip1 correlate better with the onset of G1 block than the up-regulation of p21Cip1 [47,91,210,220,221]. This, however, is subject to cell context, a striking example being a recent report that p27Kip1 is essential for the antiproliferative action of $1,25(OH)_2D_3$ on primary, but not on immortalized, mouse embryonic fibroblasts [222]. The role of CDKIs in $1,25(OH)_2D_3$–induced cell cycle arrest is also difficult to assess because, when present at relatively low levels, p21Cip1 and p27Kip1 serve to facilitate complex formation of cyclins D with Cdks, and their transport to the nucleus [223,224], and only high levels of CDKIs are inhibitory [223,225,226]. Thus, one possible explanation for the up-regulation of p21Cip1 that does not correlate with G1 arrest is that p21Cip1 simply serves to facilitate cyclin D-Cdk complex formation. More likely, however, is that elevated levels of p21Cip1 inhibit cyclin E-Cdk2 activity and block cyclin E-Cdk2 phosphorylation of p27Kip1, which leads to its degradation in proliferating cells [151,227,228].

Unlike p21Cip1, which can be directly up-regulated by $1,25(OH)_2D_3$ through a VDRE in p21Cip1 promoter [47], p27Kip1 has no VDR–binding element in its promoter, and may be regulated at both transcriptional and post-transcriptional levels, although control by protein degradation appears to be most important. While the precise mechanism of p27Kip1 up-regulation by $1,25(OH)_2D_3$ is currently not known, several reports focus on this question. One study showed that transcription factors Sp1 and NF-Y can synergistically mediate the $1,25(OH)_2D_3$–induced expression of p27Kip1 in transiently transfected U937 leukemia cells [124]. In these experiments, deletion and mutational analysis revealed that p27Kip1 promoter activation required both GGGCGG (Spl binding) and a CCAAT (NF-Y binding) sequences. As presented above (Section II.D.2.), Sp1 transcription factor is activated in $1,25(OH)_2D_3$–treated leukemia cells, so this could potentially be a plausible mechanism for the induction of G1 arrest by $1,25(OH)_2D_3$, at least in myeloid leukemia cells. However, the difficulty in accepting this scenario is that p27Kip1 mRNA levels are not found to be appreciably increased following treatment with $1,25(OH)_2D_3$ [47,229], and that in a number of cell types p27Kip1 is transcriptionally activated by the forkhead transcription factors, such as AFX (FOX04) [125,126]. More clearly related to $1,25(OH)_2D_3$–induced G1 arrest is the recent report that a deltanoid,

EB1089, inhibits the expression of the F-box protein p45Skp2 and thus prevents its degradation by the proteasome system [230]. In mouse squamous cell carcinoma AT-84 cells EB1089 did not change p27Kip1 mRNA levels, but reduced the mRNAs for p45Skp2, which ubiquitinates p27Kip1, and for Cks1, which targets p45Skp2 to p27Kip1 [230,231]. A similar decrease in p45Skp2 expression and stabilization of p27Kip1 protein was demonstrated in acute promyelocytic leukemia cells [230]. Since these changes become evident at about 48 h of the exposure to the deltanoid, there is good correlation with the onset of the G1 block. The latent period of 24–48 h for p27Kip1 up-regulation may also be needed to inactivate the cyclin E-Cdk2 complex, which phosphorylates Thr187 of p27Kip1, that is required for ubiquitination of p27Kip2 by p45Skp2 [151]. Inhibition of cyclin E-Cdk2 activity following exposure of HL60 cells to $1,25(OH)_2D_3$ has been demonstrated [204], and this may contribute to $1,25(OH)_2D_3$-induced increase in p27Kip1 levels.

b. Retinoblastoma Protein Control of Deltanoid-Induced G1 Block   The suggested placement of the inactivation of the cyclin E-Cdk2 complex upstream of p27Kip1 up-regulation raises the question of how this complex is inactivated in $1,25(OH)_2D_3$-treated cells. One possible answer is provided by the finding that the retinoblastoma (RB) gene is up-regulated early in deltanoid-induced differentiation of HL60 cells [75,98]. The increased levels of pRb can then bind and inactivate E2F transcription factors necessary for the expression of cyclin E, and thus the activity of the cyclin E-Cdk2 complex. Accordingly, the phosphorylation of T187 on p27Kip1 is reduced, allowing the accumulation of this Cdk inhibitor, and a further increase in the hypophosphorylated forms of pRb, also a substrate for the cyclin E-Cdk complex. Hypophosphorylated pRb now further binds E2F, and thus reduces cyclin E expression to the point that p27Kip1 is no longer phosphorylated and degraded, as the result of this positive feedback loop, leading to G1 arrest. It is known that in HL60 cells the expression of pRb normally occurs primarily during G1 phase [232], and can be detected at both mRNA and protein levels within 8–12 h of exposure to $1,25(OH)_2D_3$ [98], although the mechanism of its up-regulation remains to be determined. These *in vitro* studies are supported by the finding of gross defects in the development of the hematopoietic system in RB knockout mice [233,234], and by the transcriptional studies which show that the RB gene plays a role in normal human adult hematopoiesis [235]. Thus, pRb appears to have a role in the early stage of $1,25(OH)_2D_3$–induced differentiation, and contributes to changes in cellular transcriptional and kinase activities that lead to G1 arrest at a later stage.

c. Down-regulation of c-Myc Expression in Deltanoid-induced Differentiation and G1 Arrest   The pRb/E2F pathway also controls the expression of c-Myc, as E2F transcription factors have been reported to up-regulate the c-Myc gene, [236–238] and inhibition of c-Myc expression by $1,25(OH)_2D_3$ may be responsible, at least in part, for the G1 block in differentiating cells (see Figs. 4 and 6). Indeed, the association between c-Myc down-regulation and $1,25(OH)_2D_3$-induced differentiation of human leukemia cells was one of the earliest findings that initiated the studies of the molecular basis of the cellular changes that follow exposure to this hormone [16,202,239,240]. The intense interest in c-Myc as a potential negative regulator of differentiation was fueled largely by its deregulated expression in several types of human neoplasia [241,242]. c-Myc is known to promote cell cycle progression mostly through coordinated transcriptional regulation of target genes [243] (www.myccancergene.org). These include the DNA replication and cell cycle traverse-promoting genes such as ornithine decarboxylase, Cdc25A, and cyclins E and A [241]. Conversely, c-Myc inhibits the transcription of cell cycle inhibitor p21Cip1 [244], and it has been suggested that this is due, at least in part, to the sequestration of the Sp1 transcription factor, which is required for p21Cip1 transcription [245,246].

While the above considerations present an almost complete sequence of events that can explain the G1 arrest induced by $1,25(OH)_2D_3$ (summarized in Fig. 6), an additional level of control of c-Myc expression by $1,25(OH)_2D_3$ is provided by studies of Simpson *et al.* [247]. They found that in differentiating HL60 cells $1,25(OH)_2D_3$ increased the expression and DNA–binding activity of HOXB4, a product of a homeobox gene, and that HOXB4 binds to the sites in the c-Myc gene, which are involved in blocking by $1,25(OH)_2D_3$ of the elongation of c-Myc transcripts [248]. Further, these authors demonstrated that a HOXB4 antisense oligonucleotide partially inhibited the $1,25(OH)_2D_3$–induced decrease in c-Myc protein levels [249]. While they observed reduction of $1,25(OH)_2D_3$-induced differentiation in these experiments, the effect of HOXB4 antisense on the G1 block was not reported. Nonetheless, these studies are significant, as members of the HOX gene family are known to be involved in hematopoiesis and leukemogenesis [250–252]. Also, other HOX genes participate in $1,25(OH)_2D_3$–induced differentiation; HOX B7 was reported to increase in HL60 cells [253], while in U937 and MCF-7 cells $1,25(OH)_2D_3$ increased expression of HOXA10 [254].

Transcriptional blockage of c-Myc expression was also reported in colon cancer cells following exposure to $1,25(OH)_2D_3$ [255]. In these cells, c-Myc is principally under the control of APC-β catenin/T-cell

factor signaling. Thus, it is possible that $1,25(OH)_2D_3$ regulates c-Myc expression by several different pathways, but all of these appear to exert control on the c-Myc gene at transcriptional level.

2. THE G2/M RETARDATION AND POLYPLOIDIZATION

The occurrence of abnormalities in G2/M transition in $1,25(OH)_2D_3$ and other deltanoid-treated cells has been observed infrequently, with a general consensus that the G1 phase is the principal target of the antiproliferative actions of deltanoids. However, in early studies of $1,25(OH)_2D_3$ action Abe *et al*. [256] detected an increase in the G2+M compartment in WEHI murine myelomonocytic cells, also described in HL60 cells by Godyn *et al*. [257]. The basis for this increase may be a reduction in the levels of Cdk1 in these cells [258], although the roles of cohesin, separase, or Plks remain to be investigated in the light of the recently accumulating knowledge of mitotic controls (see Section III.B.3). One consequence is the higher ploidy of HL60 cells exposed for prolonged periods of time to $1,25(OH)_2D_3$, observed as an increased number of binucleated cells [257], or as nearly doubled DNA content of these cells [113]. Interestingly, polyploidization of $1,25(OH)_2D_3$-treated cells is an alternative to differentiation, as these cells over-ride the antiproliferative actions of $1,25(OH)_2D_3$ and do not express the differentiated phenotype [113]. Thus, HL60 cells can have a $1,25(OH)_2D_3$–induced defect in completion of mitosis that allows one round of DNA endoreduplication. Whether osteoclast, or perhaps megakaryocyte, polyploidization is also influenced by $1,25(OH)_2D_3$ remains a possibility.

IV. CELL-TYPE SPECIFICITY OF INHIBITION OF CELL PROLIFERATION BY DELTANOIDS WITHOUT EVIDENCE OF DIFFERENTIATION

While some effects of $1,25(OH)_2D_3$ and other deltanoids can be recognized in a variety of cell types, there is remarkable cell-type specificity in most of such effects, and it is important to realize that only a few generalizations can be made regarding the antiproliferative actions of these compounds. However, it appears to be true that deltanoid-induced differentiation is not a consequence of inhibited proliferation, as differentiation often precedes the G1 block [9,12], and deltanoids can inhibit cell proliferation with only minimal, or absent, evidence of differentiation. Indeed, the antiproliferative effect $1,25(OH)_2D_3$ on cultured melanoma cells was recognized by Colston *et al*. [259] in 1981, at

the same time as the differentiation-inducing action of $1,25(OH)_2D_3$ was described in myeloid leukemia cells by Abe *et al*. [15]. Cell specificity of responses to deltanoids is also illustrated by the finding that $1,25(OH)_2D_3$ can cause a G1 block in cultured thyroid carcinoma and pituitary corticotroph, but not lactotroph, cells [260,261]. Interestingly, while in thyroid carcinoma cells the mechanisms of the antiproliferative effects include dephosphorylation of p27Kip1 in a PTEN-dependent manner, leading to a diminished association between p45Skp2 and p27Kip1 with its consequent accumulation [260], in pituitary corticotroph cells the mechanism appears to be a diminished association of p27Kip1 with p45Skp2 and Cdk2, without an involvement of PTEN [261]. This illustrates not only the exquisite cell-type specificity of the mechanisms involved in the antiproliferative actions of deltanoids, but also that the up-regulation of p27Kip1 is unrelated to differentiation, as demonstrated previously in leukemia cells [201].

Another mechanism for the antiproliferative actions of deltanoids on cell types that show only minimal evidence of differentiation is provided by the apoptosis-inducing actions of deltanoids, as described in other chapters in this volume (e.g., Chapter 93). Again, cell type determines this response, as in contrast to various carcinomas, e.g., breast cancer cells [262], $1,25(OH)_2D_3$ protects HL60 leukemia cells from apoptosis, as first demonstrated by Xu *et al*. [263]. Thus, the activity of survival pathways may determine whether differentiation can take place in the presence of deltanoids, or whether a potential default pathway will lead to apoptosis, perhaps as the result of prolonged residence in a cell cycle compartment other than G0. In any case, deltanoids can be effective antiproliferative agents in many cell types that express VDR.

V. CONCLUSIONS

Deltanoids present new therapeutic options for treatment of human malignancies due to their demonstrated antiproliferative actions in a wide variety of cell types. While the mechanisms vary, G1 block produced by up-regulation of p27Kip1 is an almost constant feature of the cell cycle effects of deltanoids. Further studies are needed on the mechanisms that upregulate p27Kip1, although the control of its degradation by p45Skp2 that is influenced by p27Kip1-T187 phosphorylation by the E2F-cyclin E-Cdk2 pathway, and inhibition of this pathway by pRb, present exciting possibilities. Also, in view of the ability of cells treated with $1,25(OH)_2D_3$ to develop resistance to its antiproliferative actions [264,265], synergistic effects of

deltanoids combined with other agents should be further explored [266–271], as should the pathways that transmit other extracellular signals to the cell nucleus in concert with vitamin D receptor-initiated signals.

Acknowledgments

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Vitamin D and Breast Cancer

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I. INTRODUCTION

Adenocarcinoma of the breast arises when epithelial cells present in the mammary ducts or alveoli become transformed through a series of genetic and epi-genetic events. Considerable evidence indicates that estrogen, which drives mammary epithelial cell proliferation, is intricately involved in the etiology of human breast cancer. Anti-estrogens such as tamoxifen are effective for both treatment and prevention of estrogen dependent breast cancer. Tamoxifen represents the best characterized selective estrogen receptor modifier (SERM), a class of synthetic compounds that interact with the nuclear estrogen receptor (ER) in a cell type specific manner. SERMS antagonize ER signaling in breast tissue but not in bone, thus limiting proliferation of estrogen dependent breast cancer cells without adversely affecting bone mass. Although SERMs are effective in treatment of estrogen responsive disease, only one third of breast tumors are estrogen dependent, and tumor progression is often associated with loss of estrogen sensitivity. Thus, there is a need for alternative therapies that target estrogen independent cells and that minimize the progression of estrogen responsive disease to hormone independence.

Other nuclear receptors present in mammary cells, such as the progesterone receptor (PR), retinoid receptors, and the vitamin D receptor (VDR), have emerged as promising therapeutic targets for breast cancer. Based on the importance of nuclear receptors in mediating expression of genes involved in proliferation, differentiation, and apoptosis, synthetic structural analogs of nuclear receptor ligands which exhibit biological properties distinct from the natural ligands represent a feasible approach to manipulate nuclear receptor activity. Structurally, most nuclear receptor ligands have the additional advantage of being orally active. In the case of the VDR, many synthetic analogs with desirable therapeutic profiles have been developed, and some are in clinical trials for various indications, including cancer. However, further development of synthetic ligands for either treatment or prevention of breast cancer requires more accurate understanding of the role(s) of their cognate nuclear receptors in both normal and transformed mammary cells. In this chapter, we will review the extensive literature documenting the effects of $1,25(OH)_2D_3$ (the natural ligand for VDR) and numerous bio-active vitamin D analogs on breast cancer cells and tumors. Furthermore, we will highlight emerging data on the role of the vitamin D endocrine system in the normal mammary gland and the possibility that vitamin D signaling may influence breast cancer development.

II. Vitamin D Actions on Breast Cancer Cells

The VDR is expressed in the majority of human breast tumors, thus it represents a potential therapeutic target for established cancer. Extensive research has been directed towards elucidation of the effects of $1,25(OH)_2D_3$ and its synthetic analogs on breast cancer cells, and several reviews on this topic are available [1–3]. In this section, we provide a concise summary of the effects of $1,25(OH)_2D_3$ and several of its structural analogs on proliferation, differentiation, apoptosis, angiogenesis, and invasion of breast cancer cells. Potential targets of VDR signaling involved in these effects are discussed below and summarized in Table I.

A. Effects of $1,25(OH)_2D_3$ on Breast Cancer Cell Proliferation

1. CELL CYCLE PROGRESSION

Treatment of MCF-7 breast cancer cells with $1,25(OH)_2D_3$ at nanomolar concentrations induces cell

TABLE I Vitamin D and Therapy of Breast Cancer

Model	Observations
Breast cancer cells *in vitro*	• VDR expressed in human breast cancer cells; regulated by hormones and growth factors
	• 1,25(OH)$_2$D$_3$ and analogs induce G$_1$ arrest, apoptosis, and/or differentiation via VDR dependent mechanisms
	• 1,25(OH)$_2$D$_3$ suppresses estrogen, EGF, and IGF-1 mitogenic signaling and enhances growth inhibitory actions of TGFβ
	• Modulation of cell cycle regulatory proteins, oncogenes, and tumor suppressor genes linked to vitamin D mediated anti-tumor effects
	• 1,25(OH)$_2$D$_3$ induces apoptosis via caspase independent mechanisms that involve mitochondria and/or endoplasmic reticulum
	• 1,25(OH)$_2$D$_3$ inhibits angiogenesis and invasion via effects on tumor cells, endothelial cells, and extracellular matrix proteases
Animal models of breast cancer	• Vitamin D analogs inhibit growth of carcinogen-induced mammary tumors and human xenografts in absence of weight loss or hypercalcemia
	• Anti-tumor effects *in vivo* associated with induction of growth arrest and apoptosis
	• EB1089 exerts antimetastatic effects *in vivo*
Clinical Trials	• Topical calcipotriol well tolerated in women with locally advanced or cutaneous metastatic breast cancer
	• Dose escalation study indicated oral EB1089 well tolerated in majority of advanced breast cancer patients
	• Clinical effects of vitamin D analogs in advanced cancer patients to date limited to partial disease stabilization

Summary of effects of natural and synthetic vitamin D compounds on breast cancer cells *in vitro* and *in vivo*. Details and references are noted in text.

cycle arrest in G$_0$/G$_1$ [4,5], dephosphorylation of the retinoblastoma protein [5,6], and increases in the cyclin dependent kinase inhibitors p21$^{WAF-1/CIP1}$ and p27^{kip1} [7–9] (see also Chapter 92). A vitamin D responsive element (VDRE) in the human p21$^{WAF-1/CIP1}$ gene promoter suggests that p21$^{WAF-1/CIP1}$ is a direct transcriptional target of the VDR [10]. Effects of vitamin D compounds on p27^{kip1} vary with cell type; in some studies p27^{kip1} is unchanged after treatment with 1,25(OH)$_2$D$_3$ or the synthetic analog EB1089 [4,8,11], whereas in others, p27^{kip1} expression is increased [4,9,12]. Analysis of the p27^{kip1} gene promoter suggests that the 1,25(OH)$_2$D$_3$–VDR complex induces transcription of this gene through SP1 and NF-Y transcription factors rather than direct DNA binding [13].

Upregulation of p21$^{WAF-1/CIP1}$ and p27^{kip1} by vitamin D compounds is associated with inhibition of cyclin dependent kinase (CDK) activity, including CDK2 associated histone H1 kinase, cyclin D1/CDK4, and cyclin A/CDK2 [4,12]. VDR also interacts with protein phosphatases PP1c and PP2Ac to inactivate the p70 S6 kinase, which is essential for G$_1$/S phase transition [14]. Thus, the net result of vitamin D signaling is to prevent entry into S phase, leading to accumulation in G$_1$. In some breast cancer cells, vitamin D mediated G$_1$ arrest is associated with induction of differentiation markers such as lipid and casein [15–17].

2. REGULATION OF ONCOGENES AND TUMOR SUPPRESSOR GENES IN BREAST CANCER CELLS BY VITAMIN D

The effect of vitamin D compounds on MCF-7 cells has been further studied at the level of c-*myc* and c-*fos* proto-oncogene expression. EB1089 (Chapter 84) decreases c-*myc* mRNA and transiently increases c-*fos* expression, being approximately 50 times more potent than 1,25(OH)$_2$D$_3$ [16]. The observation that vitamin D signaling regulates c-*myc* mRNA is consistent with the presence of a putative VDRE in the human c-*myc* gene [18]. The p53 tumor suppressor gene plays a crucial role in regulation of growth arrest and apoptosis in response to cellular stress and DNA damage. Growth inhibition of MCF-7 cells, which express wild-type p53, by two vitamin D analogs (EB1089 and KH1060) is associated with increased p53 expression [19,20]. However, 1,25(OH)$_2$D$_3$ does not consistently up-regulate p53 in breast cancer cells [6,21], and vitamin D compounds can inhibit growth of breast cancer cells expressing mutant p53 such as T47D [22–24]. Thus, functional p53 is not required for the antiproliferative

effects of vitamin D. This notion is consistent with data indicating that $1,25(OH)_2D_3$ mediated transactivation of the p21[WAF-1/CIP1] gene promoter is p53 independent [10].

BRCA1, a tumor suppressor gene which functions in DNA repair, is also induced by $1,25(OH)_2D_3$ in MCF-7 cells [25]. BRCA1 promoter analysis indicated that the effects of $1,25(OH)_2D_3$ are indirectly mediated by the VDR. Sensitivity to $1,25(OH)_2D_3$ mediated growth inhibition correlates with induction of BRCA1 and is highest in well-differentiated breast cancer cells. These data suggest that hereditary breast cancers that develop in patients with germ line mutations in BRCA1 might be less responsive to vitamin D mediated growth inhibition.

3. ESTROGEN SIGNALING

$1,25(OH)_2D_3$ and EB1089 down-regulate ER and suppress estrogen action in MCF-7 cells [5,26–28]. Since estrogen is mitogenic for most breast cancer cells, down-regulation of estrogen regulated pathways may contribute to the antiproliferative effects of vitamin D. Sensitivity to $1,25(OH)_2D_3$ is generally higher in breast cancer cells that express ER, such as MCF-7 and T47D, than in those that do not [25,29]. Down-regulation of ER by vitamin D compounds attenuates both the mitogenic effects of estrogen and the induction of target genes such as the PR and pS2 [26,27]. Co-treatment of ER positive breast cancer cells with $1,25(OH)_2D_3$ or EB1089 and the anti-estrogens tamoxifen or ICI 182,780 inhibits proliferation more than either compound alone [26,30–33].

Sequence analysis of the ERα gene promoter has identified a potential VDRE, suggesting a direct regulatory effect of $1,25(OH)_2D_3$ on ERα gene transcription [34]. Under some conditions, $1,25(OH)_2D_3$ inhibits estrogen-induced transcription of the pS2 gene in the absence of a change in ER abundance [35], suggesting that vitamin D compounds may exert multiple effects on ER signaling. However, since $1,25(OH)_2D_3$ and its analogs also inhibit growth of estrogen-independent breast cancer cells, ER signaling is not required for the anti-tumor effects of vitamin D compounds [33,36–39]. Furthermore, in some cases, breast cancer cells selected for anti-estrogen resistance show increased sensitivity to vitamin D [40].

4. GROWTH FACTOR SIGNALING

Additional evidence suggests that vitamin D modulates secretion, processing, and/or signaling of critical growth factors in breast cancer cells. Vitamin D compounds inhibit mitogenic activity of EGF and IGF-I and induce negative growth regulators, such as TGFβ. Thus, EB1089 attenuates the growth stimulatory effects of EGF [41] and $1,25(OH)_2D_3$ regulates EGF receptor levels in breast cancer cells [42,43]. In addition, it has recently been reported that the gene encoding amphiregulin, a heparin-binding EGF-related growth factor, is transcriptionally regulated by $1,25(OH)_2D_3$ [44].

As reviewed by Sachdev and Yee [45], high plasma IGF-I is associated with increased risk of breast cancer and the IGF-I receptor (IGF-IR) is overexpressed in many breast cancer cell lines. Effects of IGF-I reflect both its extracellular concentration and the levels of IGF binding proteins (IGFBPs), which modulate its availability to the IGF-IR. In breast cancer cells, vitamin D compounds block the mitogenic effects of IGF-I, decrease expression of IGF-IR, and induce inhibitory IGFBPs such as IGFBPs-3 and -5 [37,38,46–49]. The net result of vitamin D signaling thus, is attenuation of IGF-1 stimulated mitogenesis and accumulation of IGFBPs which can promote apoptosis.

In breast cancer cells, $1,25(OH)_2D_3$ enhances the expression of the negative growth regulator TGFβ1, as well as its latent form binding protein [50,51]. A direct effect of $1,25(OH)_2D_3$ on the TGFβ2 gene is supported by the identification of VDREs in its promoter [52]. The antiproliferative effect of vitamin D compounds is partially abrogated by neutralizing antibodies to TGFβ [12,51,53,54], indicating that TGFβ can be functionally linked to the growth inhibitory effects of vitamin D *in vitro*.

B. Pro-apoptotic Effects of $1,25(OH)_2D_3$ in Breast Cancer Cells

In addition to their antiproliferative effects, $1,25(OH)_2D_3$ and its analogs induce morphological and biochemical features of apoptosis (cell shrinkage, chromatin condensation, and DNA fragmentation) in breast cancer cells [5,7,55,56]. Other markers of apoptosis induced by $1,25(OH)_2D_3$ include reorientation of phosphatidylserine (PS) to the exterior of the cell, PARP cleavage and up-regulation of apoptotic related proteins, such as clusterin, cathepsin B, and TGFβ [5,27,53,56]. Furthermore, $1,25(OH)_2D_3$ exerts additive or synergistic effects in combination with other triggers of apoptosis, such as anti-estrogens, TNFα, radiation, and chemotherapeutic agents [19,33,57–59]. It is not quite clear whether these synergistic effects result from interactions of $1,25(OH)_2D_3$ with agonist-specific signaling pathways or whether $1,25(OH)_2D_3$ impacts on components of a common apoptotic pathway.

The intracellular signaling pathways implicated in $1,25(OH)_2D_3$ mediated apoptosis of MCF-7 cells are depicted in Fig. 1. Several independent studies have

reported that sensitivity to $1,25(OH)_2D_3$–mediated apoptosis reflects the relative expression and/or subcellular localization of the Bcl-2 family of pro- and anti-apoptotic proteins. Treatment of MCF-7 cells with $1,25(OH)_2D_3$ or EB1089 induces redistribution of the pro-apoptotic Bcl-2 family member, Bax, from the cytosol to the mitochondria and down-regulates the anti-apoptotic protein Bcl-2 [5,19,60,61]. Furthermore, overexpression of Bcl-2 renders MCF-7 cells resistant to $1,25(OH)_2D_3$ mediated apoptosis [23]. Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data suggest that translocation of Bax in conjunction with down-regulation of Bcl-2 may be necessary for $1,25(OH)_2D_3$–mediated apoptosis. Vitamin D–mediated Bax translocation triggers reactive oxygen species (ROS) generation, dissipation of the mitochondrial membrane potential, and release of cytochrome c into the cytosol [60,61], features of the intrinsic (mitochondrial) pathway of apoptosis [62]. $1,25(OH)_2D_3$ also enhances mitochondrial ROS generation and cytochrome c release in MCF-7 cells treated with TNFα [63]. Of particular interest, neither Bax translocation, ROS generation, mitochondrial membrane potential dissipation, nor cytochrome c release are induced by $1,25(OH)_2D_3$ in MCF-7[DRES] cells, a variant of MCF-7 cells selected for vitamin D resistance [60,61]. Another pathway recently implicated in vitamin D–mediated apoptosis of MCF-7 cells involves calcium release from the endoplasmic reticulum and activation of μ-calpain; this process can be prevented by either calpain inhibitors or calcium buffering agents such as calbindin D28K [64]. While the specific interactions between the apoptotic pathways depicted in Fig. 1 have yet to be resolved, it is possible that signals generated from both the mitochondria and the endoplasmic reticulum cooperate to induce cell death in response to $1,25(OH)_2D_3$. It is clear, however, that MCF-7 cells undergo cell death in the presence of caspase inhibitors, indicating that the commitment to $1,25(OH)_2D_3$ mediated cell death is caspase independent [23,60].

C. Role of $1,25(OH)_2D_3$ in Regulation of Angiogenesis, Invasion, and Metastasis

Metastasis, the process by which tumor cells invade secondary sites, requires degradation of the extracellular matrix and is facilitated by angiogenesis, the growth of new blood vessels into developing tumors. Effects of vitamin D signaling on late stage breast cancer have been studied in ER negative breast cancer cell lines, such as MDA-MB-231 and SUM159PT cells, which are invasive *in vitro* and metastatic *in vivo*. In these cell
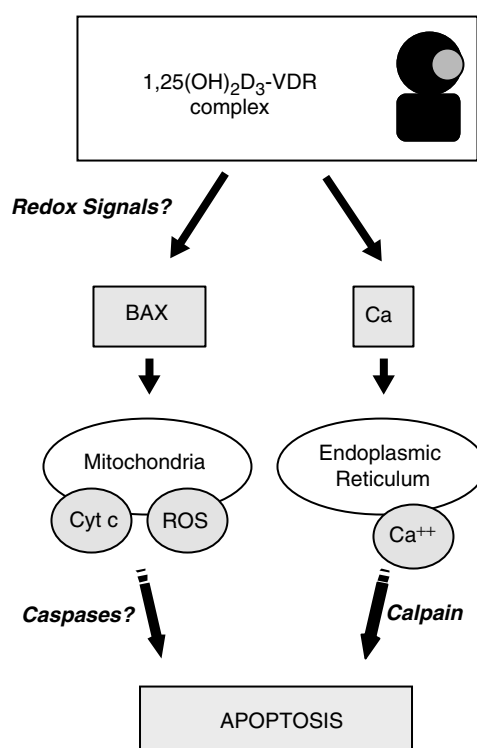
FIGURE 1   Potential mechanisms of vitamin D mediated apoptosis in breast cancer cells. VDR signaling, triggered by $1,25(OH)_2D_3$ or synthetic analogs, alter apoptotic pathways present in mitochondria and endoplasmic reticulum, leading to DNA fragmentation and cell death. The direct targets of VDR, as well as the mechanisms by which apoptosis is triggered, are not yet identified. See text for further details.

lines, $1,25(OH)_2D_3$ and EB1089 inhibit invasion as measured by the *in vitro* Boyden chamber assay [9,65]. Inhibition of invasion by vitamin D compounds can be dissociated from effects on proliferation, and may be linked to regulation of extracellular proteases such as MMP-9, urokinase-type plasminogen activator (uPA), and tissue type plasminogen activator (tPA). In MDA-MB-231 cells, these effects may result from vitamin D–mediated up-regulation of protease inhibitors PA inhibitor 1 and MMP inhibitor 1 [66].

The anti-tumor effects of $1,25(OH)_2D_3$ may also involve regulation of angiogenesis, since $1,25(OH)_2D_3$ inhibits angiogenesis in the chick embryo chorioallantoic membrane assay [67] and in tumor cell-induced angiogenesis assays in mice [68]. Moreover, vitamin D analogs reduce angiogenesis of MCF-7 breast tumors overexpressing vascular endothelial growth factor (VEGF) and inhibit VEGF expression in MDA-MB-231 xenografts [69,70]. VDR is expressed in endothelial cells [71] and $1,25(OH)_2D_3$ blocks both basal and VEGF-induced endothelial cell sprouting, elongation, and proliferation [70,72]. Collectively, these studies

indicate that vitamin D signaling likely inhibits angiogenesis via VDRs expressed on both the transformed mammary epithelial cells and the endothelial cells within breast tumors.

III. DETERMINANTS OF BREAST CANCER SENSITIVITY TO VITAMIN D

A. Ligand Availability

Circulating $1,25(OH)_2D_3$ is delivered to cells via the serum vitamin D–binding protein, but little is known about metabolism or half life of $1,25(OH)_2D_3$ in breast cancer cells. Catabolism of $1,25(OH)_2D_3$ is initiated via hydroxylation at the 24 position in the side chain, a reaction catalyzed by the $25(OH)D_3$ 24-hydroxylase (CYP24). Comparative genome hybridization studies have found that CYP24 is amplified in human breast cancer [73], suggesting that enhanced catabolism of $1,25(OH)_2D_3$ by the 24-hydroxylase, leading to reduced ligand availability to the VDR, could contribute to breast cancer. In addition to uptake of $1,25(OH)_2D_3$ from the circulation, it is formally possible that breast cancer cells express the $25(OH)D_3$ 1α-hydroxylase (CYP27B1) and produce $1,25(OH)_2D_3$ from $25(OH)D_3$. The 1α-hydroxylase mRNA has been detected in MCF-7 cells and in human breast tumors [74]; however, MCF-7 cells are not growth inhibited by $25(OH)D_3$ [75], suggesting that access to or activity of the 1α-hydroxylase enzyme is not sufficient to generate growth-inhibitory concentrations of $1,25(OH)_2D_3$. In contrast, nontransformed human mammary epithelial cells express 1α-hydroxylase and are growth inhibited by physiological concentrations of $25(OH)D_3$ [75], indicating that 1α-hydroxylase activity may be physiologically relevant in normal breast cells. Further studies to assess the metabolism of both $25(OH)D_3$ and $1,25(OH)_2D_3$ in mammary cells as a function of transformation will be necessary to clarify the role of 1α-hydroxylase in breast cancer.

B. Expression and Regulation of VDR in Breast Cancer Cells

Receptors for $1,25(OH)_2D_3$ have been demonstrated in carcinogen-induced rat mammary tumors, human breast tumors and established breast cancer cell lines [29,76,77]. A recent study has clarified the importance of the nuclear VDR in mediating the effects of $1,25(OH)_2D_3$ and its analogs in breast cancer cells. Zinser *et al.* [78] utilized cell lines derived from carcinogen-induced mammary tumors generated in VDR knockout mice and wild-type control litter mates to

demonstrate that cells lacking VDR fail to respond to vitamin D compounds. In contrast VDR expressing mammary tumor cell lines generated from tumors that developed in wild-type mice were growth inhibited. These data confirm that the nuclear VDR is required to mediate anti-tumor effects and therefore, expression, function, and regulation of VDR in mammary cells are important determinants of sensitivity to vitamin D.

VDR abundance is affected by many physiological factors and is achieved through a variety of mechanisms, including alterations in transcription and/or mRNA stability, post-translational effects, and ligand-induced stabilization. Expression of the VDR in cultured cells and *in vivo* is regulated by many physiological agents, including $1,25(OH)_2D_3$ itself, estrogens, retinoids, and growth factors [79–81]. Thus, breast cancer cell sensitivity to $1,25(OH)_2D_3$ mediated growth regulation may in part reflect the activity of other hormone signaling pathways through their impact on VDR expression. Comparison of a panel of breast cancer cells indicates that ER positive cells tend to express higher levels of VDR than ER negative cells [29]. Furthermore, *in vitro* studies demonstrate that estrogen up-regulates the VDR, and anti-estrogens such as tamoxifen down-regulate the VDR, in ER positive breast cancer cells [75,82]. In MCF-7 and T47D cells, estrogen transcriptionally up-regulates the VDR promoter upstream of exon 1c [24,83]. Collectively, these data support the concept that estrogen and anti-estrogens are important regulators of VDR expression in breast cancer cells, a concept with clinical implications arising from the potential use of SERMs for prevention and/or treatment of breast cancer and osteoporosis. The efficacy and toxicity of vitamin D analogs is determined, in part, by the level of VDR in target tissues, and thus it will be important to determine the degree to which estrogen status influences VDR abundance in different $1,25(OH)_2D_3$ target cells *in vivo* (i.e., breast, bone, uterus). In this respect, it will also be important to assess whether novel SERMs or phytoestrogens currently utilized by post-menopausal women act as estrogen agonists or antagonists in regulation of VDR expression. The phytoestrogen resveratrol has recently been shown to up-regulate VDR and sensitize breast cancer cells to vitamin D–mediated growth inhibition, offering proof of principle that dietary factors can impact on cellular sensitivity to $1,25(OH)_2D_3$ through regulation of VDR [24].

C. Vitamin D Resistance

Although it is clear that the VDR is required for breast cancer cell responsiveness to vitamin D compounds,

a number of established breast cancer cell lines that express VDR fail to respond to the anti-proliferative effects of $1,25(OH)_2D_3$. Data from mammary cell lines suggest that oncogenic transformation with SV40 or ras inhibits VDR signaling and induces resistance to the growth inhibitory effects of $1,25(OH)_2D_3$ [80,84], raising the possibility that breast cancer progression may be facilitated by deregulation of the vitamin D pathway.

In an effort to understand the cellular basis for insensitivity to vitamin D, Narvaez *et al.* [82] selected and characterized $1,25(OH)_2D_3$–resistant subclones of MCF-7 cells. The resulting MCF-7^{DRES} cells express VDR, but do not undergo growth arrest or apoptosis in response to $1,25(OH)_2D_3$. MCF-7^{DRES} cells are selectively resistant to $1,25(OH)_2D_3$ and its structural analogs, and respond to other anti-proliferative agents [21,33,82]. Similar results have been obtained in an independently derived $1,25(OH)_2D_3$–resistant subclone of MCF-7 cells, labeled MCF-7/VDR [8]. The mechanisms underlying vitamin D resistance in these MCF-7 clones are incompletely understood. Theoretically, selective insensitivity to $1,25(OH)_2D_3$ could be secondary to defective VDR, reduced availability of ligand, or uncoupling of a functional vitamin D signaling pathway from growth arrest/apoptosis. While resistance could be associated with elevated expression of the vitamin D 24-hydroxylase enzyme which inactivates $1,25(OH)_2D_3$, this does not appear to be the case for either of the vitamin D–resistant MCF-7 variants. Both MCF-7^{DRES} and MCF-7/VDR cells contain transcriptionally active VDRs when measured with consensus VDREs; however, basal VDR expression is lower in both resistant cell lines than in parental MCF-7 cells. In MCF-7^{DRES} cells, $1,25(OH)_2D_3$ comparably up-regulates the steady state level of the VDR protein in both sensitive and resistant cell lines [21]. MCF-7^{DRES} cells can be sensitized to the growth-inhibitory effects of $1,25(OH)_2D_3$ by co-treatment with low concentrations of the phorbol ester TPA, suggesting that phosphorylation pathways may be altered in this cell line [21,61]. Further studies with these interesting cell lines will be necessary to resolve the mechanism(s) of vitamin D resistance. Significantly, the MCF-7^{DRES} cell line retains resistance to vitamin D analogs when grown as xenografts in nude mice [85], providing an important model system for understanding the basis of vitamin D resistance *in vivo*.

D. Prognostic Significance of Breast Tumor VDR Expression

A high proportion (>80%) of breast cancer biopsy specimens contain VDR [86–88], and up-regulation of VDR protein in breast carcinomas compared to normal breast tissue has been reported [89]. In breast tumors, there is no significant correlation between VDR expression and ER expression, lymph node status, or tumor grade [90,91]. Tumor VDR status does not appear to be related to overall survival [86,87,91] or to survival after relapse [91]. However, in a study of 136 patients with primary breast cancer, it was found that women with VDR negative tumors relapsed significantly earlier than women with VDR positive tumors [91,92].

IV. VITAMIN D ANALOGS: PRECLINICAL AND CLINICAL TRIALS

A. Natural Ligands Versus Synthetic Analogs

While the beneficial effects of $1,25(OH)_2D_3$ on cancer cells support its use as a therapeutic agent, natural vitamin D metabolites exert potentially toxic effects on calcium handling at the doses required for anti-tumor effects. Thus, the metabolite $1\alpha(OH)D_3$, which is converted to $1,25(OH)_2D_3$ *in vivo*, effectively inhibits tumor growth *in vivo*, but the therapeutic window is extremely narrow [reviewed in 2]. Modifications to the parent vitamin D structure have successfully generated synthetic analogs with enhanced growth regulatory effects and limited calcium mobilizing action (see Section VIII of this book). Several vitamin D analogs have been tested in preclinical and clinical models of breast cancer as described below.

B. Effects of Vitamin D Analogs on Breast Cancer Cells and Tumors

1. CALCIPOTRIOL

Calcipotriol (MC903, Leo Pharmaceutical Products, Denmark) contains a cyclopropyl substitution in the side chain [93] and is equipotent with $1,25(OH)_2D_3$ in inhibition of MCF-7 cell growth *in vitro* [94]. However, due to rapid inactivation of the analog *in vivo*, calcipitriol displays calcemic activity 100–200 times less than $1,25(OH)_2D_3$. This compound is marketed for topical treatment of psoriasis (see Chapter 101).The efficacy of topical treatment with calcipotriol was assessed in 19 women with locally advanced or cutaneous metastatic breast cancer [95]. Of 14 patients who completed treatment with topical calcipotriol ointment, three showed partial response and one exhibited a minimal response.

2. MAXACALCITOL (OCT)

22-oxa-1,25(OH)$_2$D$_3$ (Chugai Pharmaceutical Co. Ltd), in which an oxygen atom is substituted for the methyl group at C-22 is described in detail in Chapter 86. OCT displays reduced calcemic activity *in vivo* and effectively inhibits growth of both ER positive MCF-7 xenografts and ER negative MX-1 tumors in nude mice [36]. In MCF-7 xenografts, OCT exerts synergistic antitumor effects with the anti-estrogen tamoxifen [30]. Furthermore, OCT exerts growth-inhibitory effects on dimethylbenzanthracene (DMBA)-induced rat mammary tumors, alone and in combination with the aromatase inhibitor CGS 16949A [96], and reduces VEGF expression in MDA-MB-231 breast tumors [69].

3. SEOCALCITOL (EB1089)

Seocalcitol (EB1089), a second generation analog from Leo Pharmaceutical Products is detailed in Chapter 84. EB1089 contains a conjugated double-bond system and is approximately 50 times more potent than 1,25(OH)$_2$D$_3$ *in vitro* with markedly reduced effects on calcium metabolism *in vivo* [16,94,97]. Oral administration of EB1089 to rats bearing nitrosomethyl urea (NMU)-induced mammary tumors dose-dependently inhibits tumor growth, and effective low doses do not increase serum calcium [94,98]. Anti-tumor effects of EB1089 are also observed in DMBA-induced mammary tumors and MCF-7 xenografts [41,85]. In both the NMU-induced tumor model and MCF-7 cell xenografts, EB1089 induces tumor regression through inhibition of proliferation and induction of DNA fragmentation indicative of apoptotic cell death [85,99]. The beneficial effects of EB1089 on tumor progression in the MCF-7 xenograft model is enhanced when co-administered with paclitaxel [100], retinoic acid [101], or radiation [59]. EB1089 also inhibits development of bone metastases and increases survival of mice following intracardiac inoculation of ER negative MDA-MB-231 cells [102].

Based on a dose-finding study in 13 healthy volunteers, doses in the range of 5–20 μg/day EB1089, given orally for four consecutive days, can be considered for future use in clinical trials. A phase I trial of oral EB1089 in patients with advanced breast and colorectal cancer has been completed. This trial was an open, noncontrolled single-center study with sequentially assigned dose levels [103]. Twenty-five women with breast cancer and four women and seven men with colorectal carcinoma received EB1089 twice daily for five days with a three week post-dosing follow-up. Twenty patients received compassionate treatment after this post-dosing interval for between 10 and 234 days (mean 90±62 days). On the basis of this study, the estimated maximum tolerated dose of EB1089 for prolonged use is approximately 7 μg/m²/day. Ten patients developed

hypercalcemia, which resolved by seven days after cessation of treatment, and no other serious adverse reactions were observed. Although no clear anti-tumor effects were seen in this study, six patients (two colorectal, four breast cancer) showed disease stabilization for at least three months.

4. OTHER VITAMIN D ANALOGS WITH ANTI-TUMOR EFFECTS ON BREAST CANCER

The 16-ene vitamin D analogs are characterized by the introduction of a double bond at the C16 position in the D ring of the molecule [104] (see Chapter 85). 1α,25-dihydroxy-16-ene-23-yne-cholecalciferol (Ro23-7553 or ILX-23-7553, Hoffmann-LaRoche Ltd) is more potent than 1,25(OH)$_2$D$_3$ in cell growth inhibition and is currently in phase I trials for patients with advanced metastatic cancer.

The Hoffmann-LaRoche compound Ro25-6760 is a 19-nor-hexafluoride analog that suppresses growth of human breast cancer cells *in vitro* and inhibits growth of MCF-7 xenografts in nude mice [105]. Synergistic inhibition of tumor growth is observed in animals given Ro25-6760 in combination with paclitaxel [100]. The 20-epi analogs, including 20-epi(S)-ethoxy-23-yne 24a,26a,27a-trihomo1α,25-dihydroxyvitamin D$_3$ (CB1093, Leo Pharmaceuticals) and 20-epi-22oxa-24a,26a,27a-tri-homo-1,25(OH)$_2$D$_3$ (KH1060, Leo Pharmaceuticals), potently inhibit growth of breast cancer cells, xenografts, and NMU-induced rat mammary tumors [20,106,107]. Novel analogs with 19-nor and 14-epi modifications developed by Bouillon and colleagues also exhibit anti-cancer effects in human breast cancer cells *in vitro* and *in vivo* [108]. The analog 1α-hydroxy-24-ethyl-cholecalciferol (1α(OH)D$_5$) developed by Mehta and colleagues exerts anti-tumor effects against established human breast cancer cells and directly inhibits preneoplastic lesion development in mouse mammary gland organ culture [109]. Collectively, these studies offer proof of principle that vitamin D analogs can inhibit breast cancer progression with minimal calcemic side effects. While it is clear that most, if not all, vitamin D analogs mediate their growth-inhibitory effects through the VDR [78], further mechanistic studies are required to understand the selective actions of these analogs *in vivo* (see Chapters 82 and 83).

V. VITAMIN D AND PREVENTION OF BREAST CANCER

A. Expression and Role of VDR in Normal Mammary Gland

A potential role of vitamin D in breast cancer prevention has been suggested based on animal,

TABLE II Evidence Linking Vitamin D to Prevention of Breast Cancer

Approaches	Observations
Animal models	• VDR is expressed in normal mammary gland
	• 1,25(OH)$_2$D$_3$ inhibits hormone stimulated ductal growth and branching
	• 1,25(OH)$_2$D$_3$ inhibits carcinogen induced pre-neoplastic lesions in mammary organ culture
	• VDR null mice exhibit accelerated mammary gland development
Epidemiological studies	• Inverse associations reported between biomarkers of sunlight exposure, dairy products, and/or dietary vitamin D and risk of breast cancer
	• Low serum 1,25(OH)$_2$D$_3$ associated with enhanced breast cancer risk and/or disease activity
Genetic studies	• Amplification of 25-hydroxyvitamin D 24-hydroxylase in breast cancers
	• VDR polymorphisms linked to breast cancer risk and/or metastatic progression
	• Fok 1 and singlet A repeat polymorphisms affect VDR transcriptional activity

Summary of data generated in animal, epidemiological, and genetic studies, see text for details and references.

epidemiological, and genetic studies (Table II). The VDR is present in rabbit, rat, mouse, and human mammary gland [110–113], and its expression is developmentally regulated. VDR expression is high throughout puberty, pregnancy, and lactation, periods of maximal tissue growth and remodeling [78,113,114]. The dynamic regulation of VDR in mammary gland during the reproductive cycle suggests that hormones and/or growth factors that impact on glandular development may modulate VDR expression. Indeed, lactogenic hormones up-regulate VDR in normal mammary gland and nontranformed mammary cells *in vitro* [80,115]; however, the specific factors responsible for VDR regulation in the normal mammary gland *in vivo* have yet to be defined.

Developmental regulation of VDR in mammary cells implies that vitamin D signaling may be involved in the regulation of glandular function. *In vitro*, 1,25(OH)$_2$D$_3$ inhibits growth of nontransformed mammary cells as well as breast cancer cells [75,80,81]. However, in contrast to breast cancer cells, nontransformed mammary cells exhibit markers of differentiation rather than apoptosis [15,80,116]. This has led to the suggestion that 1,25(OH)$_2$D$_3$ and the VDR induce a program of genes that inhibit proliferation and maintain differentiation in the normal gland [117]. This suggestion is supported by organ culture studies that demonstrate effects of 1,25(OH)$_2$D$_3$ on calcium transport, casein expression, and branching morphogenesis [113,118,119]. Furthermore, mammary glands from VDR-ablated mice are heavier and exhibit increased ductal extension and branching morphogenesis compared to glands from wild-type control mice [113]. In addition, glands from VDR-ablated mice exhibit enhanced growth in response to estrogen and progesterone, both *in vivo*

and in organ culture, compared to glands from control mice. In organ culture, 1,25(OH)$_2$D$_3$ inhibits branching of mammary glands from control mice but has no effect on glands from VDR knockout mice. These and other data, reviewed in Welsh *et al.* [81], provide evidence that 1,25(OH)$_2$D$_3$ and the nuclear VDR exert growth inhibitory effects on normal mammary cells during early development of the gland.

B. Prevention of Breast Cancer by Vitamin D: Preclinical Studies

Identification of 1,25(OH)$_2$D$_3$ and the VDR as components of a signaling network that impacts on proliferation and differentiation in the normal mammary gland raises the possibility that optimal vitamin D status may protect against mammary transformation. In support of this suggestion, rats fed diets high in calcium and vitamin D develop fewer mammary tumors in response to the carcinogen dimethylbenzanthracene (DMBA) than mice fed diets low in calcium and vitamin D [120]. However, whether this difference specifically reflects vitamin D signaling is unclear since vitamin D deficiency is associated with multiple metabolic disturbances. Prevention of NMU-induced mammary tumors with vitamin D analogs, including Ro24-5531 [1α,25-dihydroxy-16-ene-23-yne-26-27-hexafluorocholecalciferol) and 1α(OH)D$_5$ provide further support that vitamin D may protect against breast cancer [121,122]. A direct effect of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ on the sensitivity of the mammary gland to transformation is suggested by studies indicating that both vitamin D compounds prevent DMBA induced preneoplastic lesions in organ culture [123].

C. Epidemiological Studies on Vitamin D Status and Breast Cancer

The majority of women who develop breast cancer are of postmenopausal age, and estrogen deficiency and aging are often associated with vitamin D deficiency. However, few epidemiological studies have examined whether dietary intake of vitamin D *per se* alters breast cancer incidence in populations (see Chapter 91 for a discussion). An evaluation of the Nurses Health Study [124] found that intakes of dairy products, dairy calcium, and total vitamin D (as measured by food frequency questionnaires) were inversely associated with breast cancer risk in premenopausal, but not postmenopausal, women. These data are consistent with an earlier study that reported an inverse correlation between intake of dairy products and breast cancer risk [125]. Another recent study included evaluation of sunlight exposure in addition to vitamin D from diet and supplements in relation to breast cancer risk [126]. In this study, several measures of sunlight exposure and dietary vitamin D intake were associated with a reduced risk of breast cancer; however, the associations were dependent on region of residence. Correlations between risk of breast cancer and exposure to solar radiation, which increases epidermal synthesis of vitamin D, have also been proposed [127–129] (see Chapter 90). In two studies where vitamin D status was measured in relation to breast cancer, low levels of $1,25(OH)_2D_3$ were found to be associated with increased breast cancer risk or disease progression [130,131].

D. VDR Polymorphisms and Breast Cancer Risk

There has been considerable interest in genetically determined differences in the VDR signaling pathway in relation to disease susceptibility. A number of common allelic variants, or polymorphisms, in the human VDR gene have been examined in relation to risk of breast cancer. The best studied VDR polymorphisms include a start codon polymorphism (*FokI*) in exon 2, *BsmI* and *Apa I* polymorphisms in an intronic region between exons VIII and IX, a *Taq I* variant in exon IX and a singlet (A) repeat in exon IX. An Australian study [132] examined *Apa* I and *Taq* I polymorphisms in patients with breast cancer compared to women with no history (family or personal) of breast cancer. Allele frequencies of the *Apa* I polymorphism showed a significant association with breast cancer risk while the *Taq* I polymorphism showed a similar trend, but the association was not significant, and allelle frequencies

of the *Fok* I polymorphism were not significantly different. In a Japanese population study on the *Bsm* I polymorphism, the bb genotype conferred an almost fourfold increase in the risk of breast cancer [133]. A study in a London Caucasian population also demonstrated a significant association between breast cancer risk and the *Bsm* I polymorphism, with the odds ratio for bb vs BB genotype over 2.3 [134]. The 'L' poly(A) variant was also associated with a similar risk in this study. However, the data are not entirely consistent, as two reports showed no association between the *Taq* I polymorphism and breast cancer risk [135,136], and an increased (rather than decreased) breast cancer risk was associated with the BB genotype among Latina women in the United States [137] and with the AA genotype in a small study of Taiwanese women [138]. Other studies have shown an association of VDR polymorphisms with disease progression rather than breast cancer risk. Ruggiero *et al.* [139] suggested that the VDR *Bsm* I polymorphism was related to development of more aggressive metastatic breast cancer, and a second study reported a significant association between the bb VDR genotype and presence of lymph node metastases [140]. Schondorf *et al.* [141] reported that breast cancer patients with the AA genotype have a 1.7-fold increased risk of developing bone metastases, whereas patients with the TT genotype have a 0.5-fold risk.

Although these findings are certainly intriguing, the underlying basis for an association between VDR polymorphisms and breast cancer susceptibility is currently unclear. Three of the VDR polymorphisms that have been linked to breast cancer susceptibility (*BsmI*, *Apa I*, or *Taq I* variants) do not alter the amount, structure, or function of the VDR protein produced. There is evidence, however, that two VDR polymorphisms (the VDR start codon polymorphism defined by *FokI* and the singlet (A) repeat in exon IX) may have functional significance. The *FokI* site dictates which of two potential translation initiation sites is utilized. Individuals lacking the *FokI* restriction site initiate translation at the first site, and express the full length VDR consisting of 427 amino acids. In contrast, individuals with the *FokI* restriction site utilize a second ATG site, generating a VDR protein of 424 amino acids. Although no significant differences in ligand affinity, DNA binding, or transactivation activity were found between these two VDR forms when studied independently, when the VDR start codon polymorphism was considered simultaneously with the singlet (A) repeat in exon IX, differences in VDR function were detected *in vitro* [142]. In transient transfection assays with a vitamin D responsive reporter gene, the shorter VDR variant was shown to interact more strongly with the

transcription factor TFIIB and display higher potency than the longer VDR variant. These data support the concept that functionally relevant polymorphisms in the VDR exist, and further studies will be required to determine whether VDR genotype interacts with other risk factors for breast cancer (also see Chapter 68).

VI. SUMMARY AND OUTSTANDING RESEARCH QUESTIONS

VDR and $1,25(OH)_2D_3$, its natural ligand, act through multiple signaling pathways to induce growth arrest, differentiation, and apoptosis in mammary epithelial cells (Fig. 2). Synthetic analogs of $1,25(OH)_2D_3$, which have

potent growth inhibitory effects with minimal calcemic activity *in vivo*, provide proof of principle that vitamin D signaling can inhibit the growth of established tumors in animal models. Studies with VDR null mice indicate a functional role for vitamin D signaling in the normal mammary gland. Clinical studies and epidemiological approaches have provided evidence that vitamin D signaling represents a target for breast cancer prevention. Challenges for the future include better understanding of the transport, uptake, and metabolism of $1,25(OH)_2D_3$ and bioactive analogs in breast cancer cells, the molecular mechanism of action and specific targets of the VDR in mammary gland, and the influence of genetic differences in the VDR on an individual's response to vitamin D compounds. Such understanding should provide insight into design of vitamin D–based strategies to impact on breast cancer development or therapy.
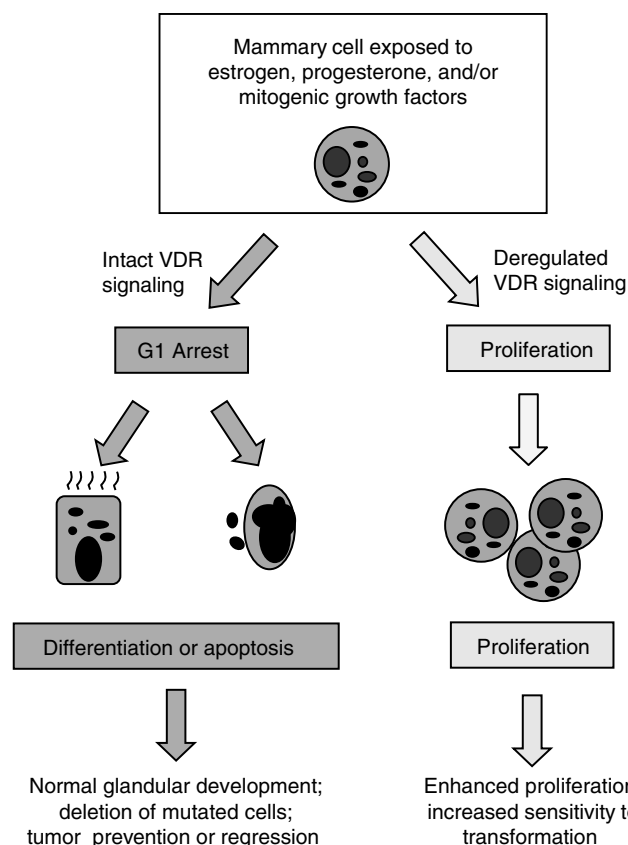

FIGURE 2   Model for effects of VDR pathway on normal or transformed mammary epithelial cells. In the presence of functional VDR, $1,25(OH)_2D_3$ attenuates hormone and growth factor stimulated proliferation, inducing G1 arrest and differentiation in normal cells and/or apoptosis in transformed cells. Thus, vitamin D signaling is predicted to maintain normal glandular development, eliminate mutated cells, and/or limit growth of established tumors. In the absence of VDR signaling, cells are more sensitive to the mitogenic effects of hormones and growth factors and may be more likely to undergo transformation.

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Vitamin D and Prostate Cancer

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I. INTRODUCTION

A. Scope of the Problem

Adenocarcinoma of the prostate gland is the most commonly diagnosed malignancy in American men, excluding skin cancer [1]. The number of cases may actually be substantially underestimated since clinically silent prostate cancer (PCa) is very common. In men over the age of 50, subclinical PCa is found in as many as 40% of individuals [2]. Although PCa is generally a slow growing malignancy, mortality from this disease is nonetheless considerable. In contrast to many other malignancies, the incidence of PCa has continued to rise each year and currently PCa is the second leading cause of cancer death among U.S. men [3]. Over the last two decades the age-adjusted mortality rate from PCa increased 7% among U.S. Caucasian men. Since PCa rates increase with advancing age, one can expect that PCa will become an even greater problem as life expectancy continues to increase. As a result, PCa has rapidly become a major public health concern not only in the U.S. but also worldwide.

B. Etiology, Treatment, and the Role of Hormonal Factors

The etiologic factors associated with PCa are varied and include age and race as well as genetic, dietary, and hormonal influences [4]. Androgens are integrally involved in the regulation of prostate growth and cell proliferation [5]. Although several studies have demonstrated a correlation between serum testosterone levels and increased risk of PCa [6], it is unclear if androgen plays a dominant role in neoplastic transformation [5]. Circulating androgens do promote tumor growth and perhaps participate in neoplastic transformation. Indeed, the withdrawal of androgens causes involution and apoptosis of normal as well as malignant prostatic epithelial cells [6,7]. This provides the basis for the use of androgen ablation in the treatment of clinically advanced PCa and androgen deprivation therapy remains the mainstay of PCa treatment [3].

At the outset of this chapter, a brief overview of therapeutic considerations would be useful. Early diagnosis of PCa is often made by screening for prostate specific antigen (PSA), one of the most useful tumor markers. A digital rectal exam (DRE) may also indicate prostate enlargement and/or abnormal texture. When indicated, prostate biopsy is performed to confirm the presence of cancer. If the cancer is felt to be confined to the prostate, therapeutic choices for primary therapy are prostatectomy or radiation using external beam or radioactive seed implantation. Elderly men, especially with less invasive pathology on biopsy or those with comorbidities, may be watched (watchful waiting) with monitoring of PSA levels. If the cancer is restricted to the prostate capsule, surgery or radiation may lead to a cure. If the cancer had already escaped the capsule, primary therapy would fail and eventually the PSA would again be found to rise indicating that the cancer had spread. Androgen ablation therapy almost always leads to cancer regression, whether treating cancer recurrence following primary therapy or in those men with metastatic cancer. Deprivation of androgens can

be accomplished by orchiectomy or more commonly today by pharmacological means using gonadotropin releasing hormone (GnRH) analogs such as lupron, zoladex, or other drugs that inhibit luteinizing hormone (LH) release. Additional therapy may include the use of anti-androgen drugs (androgen antagonists), such as flutamide or casodex, that block binding of androgens to the androgen receptor (AR). A recent trial suggested that the 5α-reductase inhibitor finasteride might reduce prostate cancer incidence, although the cancers that did occur might be more aggressive [8]. Initially, almost all patients respond to andorogen ablation therapy. However, many if not all patients eventually fail as the cancer develops the ability to grow in the absence of androgens [9,10]. This is called androgen-independent prostate cancer (AIPC) or hormone refractory prostate cancer (HRPC) [3]. AIPC is the progressive and metastatic form of the disease and unfortunately, it is not amenable to current therapies. This transition of PCa to AIPC remains both a therapeutic as well as an experimental challenge.

Growth of the normal or malignant prostate is dependent on androgens. Circulating testosterone is converted to the more potent dihydrotestosterone (DHT) within the prostate by the enzyme 5α-reductase. Both testosterone and DHT act via classical AR to stimulate growth, cell survival, PSA, and other androgen-regulated genes. Most cases of AIPC retain AR. There are several molecular mechanisms underlying AIPC development including: a) amplification of the AR gene, which increases AR sensitivity to even low levels of androgens; b) mutations in the AR gene that broaden ligand specificity so that nonandrogens or even androgen antagonists can stimulate prostate growth; c) androgen-independent activation of the AR by growth factor signaling pathways or activation of AR coactivators; and d) pathways that are independent of AR [10].

Evidence is now accumulating for the etiologic role of AR gene mutations in the pathogenesis of some cases of AIPC [9–11]. The presence of missense mutations in the AR gene renders the AR "promiscuous" and allows the inappropriate activation of the AR by nonandrogen steroids as well as AR antagonists leading to AIPC [9–16]. Nonandrogen steroid hormones such as estrogens, progestins, as well as glucocorticoids may activate AR containing mutations in its ligand-binding domain and thereby stimulate the growth of PCa cells that harbor such promiscuous mutant ARs [17–19]. Some of these AR mutations also enable AR antagonists to acquire agonist activity leading to a failure of androgen ablation therapy [20,21].

Other steroid hormones, such as progestins and estrogens, may play a role in PCa. Receptors for both progesterone and estrogen have been observed in prostatic tumors [22]. The presence of estrogen receptor (ER)-α and -β has been demonstrated in normal prostate, dysplasia, and cancer [23]. Estrogens and selective estrogen receptor modulators (SERMs) have been shown to modulate the growth and even induce apoptosis in PCa cell lines [24]. Estrogens have been shown to stimulate the growth of LNCaP, human PCa cells [25]. A selective ER antagonist ICI 182,780 (faslodex) caused growth inhibition in the human PCa cell lines PC-3 and DU 145, and this growth inhibition was abrogated by an ER anti-sense oligonucleotide [26]. Phytoestrogens, such as genistein, daidzein, and equol, also inhibited the growth of human prostate cancer cells [27]. However, in addition to acting through their own receptors, estrogens, SERMs, progestins, glucocorticoids, and other steroid hormones can activate mutated ARs in PCa cells or tumors harboring promiscuous AR mutations, and thereby modulate cancer cell growth [10].

One of the goals of current research on PCa and AIPC is the identification of new agents that would prevent and/or slow down the progression of this disease and in recent years vitamin D has emerged as a promising therapeutic agent [28–34]. We describe in the following sections several lines of evidence for the potential benefits of vitamin D in PCa.

C. Epidemiology

1. SUNLIGHT EXPOSURE

Based upon epidemiological studies, several risk factors for prostate cancer have been identified including age, race, and genetic factors [7,35]. Environmental factors may also have a strong influence upon the expression of the disease. For example, when compared to Nigerian men, African American men have a sixfold increased risk of developing clinically detectable prostate cancer [7]. It has long been appreciated that solar radiation can decrease the mortality rates of non-cutaneous malignancies [36,37]. Age is the strongest risk factor for prostate cancer, and the elderly are frequently vitamin D–deficient due to several factors, including less exposure to UV radiation (see Chapter 3). Of particlular interest is the hypothesis put forward by Schwartz and colleagues [38,39] suggesting a role for vitamin D in decreasing the risk of developing prostate cancer (see Chapter 90). Their hypothesis is based upon the observation that prostate cancer mortality rates in the U.S. are inversely proportional to the geographically determined incident UV radiation exposure from the sun, and that UV light is essential for vitamin D synthesis. This hypothesis is not without precedent, as vitamin D may have a role in the prevention of colon cancer as well (see Chapter 95). It may also offer a

potential explanation of why African American men have a higher incidence of prostate cancer than Caucasian men [40]. African American individuals have lower serum 25-hydroxyvitamin D [25(OH)D] levels as a result of their darker skin pigmentation because the high melanin levels in darkly pigmented skin block UV radiation and inhibit the formation of vitamin D_3 [41] (see Chapters 3 and 47). The findings of a recent study of patients with PCa and benign prostatic hyperplasia (BPH) [42] suggest that susceptibility to PCa is in part determined by the extent of exposure to UV radiation and that the ability to pigment the skin modulates this effect.

2. DIET AND SERUM LEVELS OF VITAMIN D METABOLITES

Dietary forms of vitamin D include vitamin D–supplemented milk and other foods, ergocalciferol (vitamin D_2) in plants, and vitamin D_3 in animal products. Diet has been proposed as a risk factor for PCa, and the low risk of PCa for indigenous Japanese has been postulated to be related to their traditional diet [43]. This diet, among other attributes, is rich in oily fish, which are an important dietary source of vitamin D_3. Some epidemiological studies show that high levels of dietary calcium are a significant risk factor for prostate cancer [44,45] (see Chapter 91). This may be relevant to the relationship of vitamin D and PCa because high levels of serum calcium suppress parathyroid hormone and reduce the renal production of 1,25-dihydroxy-vitamin-D, $(1,25(OH)_2D_3)$. One of these studies [44] also shows that a high intake of fruit is associated with a decreased risk for PCa. The authors suggest that high intake of fruit-derived fructose would lead to hypophosphatemia, which stimulates $1,25(OH)_2D$ production. These observations provide indirect support for the possible protective role of high $1,25(OH)_2D$ levels on PCa. However, another recent study does not find an association between vitamin D intake and PCa risk in a population of American men [46].

Corder *et al*. [47] undertook a case-control study and determined the levels of vitamin D metabolites in stored sera collected in the San Francisco Bay area between 1964 and 1971 and matched for age, race, and day of serum storage. This study looked at a nested group of sera from men who ultimately developed PCa out of a larger population of 250,000 individual samples. Mean levels of $1,25(OH)_2D$, the active metabolite of vitamin D, were slightly but significantly lower in men who went on to develop PCa when compared to controls who did not develop cancer. The risk of PCa decreased with higher $1,25(OH)_2D$ concentration, especially in men with low 25(OH)D. In men over the age of 57, serum $1,25(OH)_2D$ concentration was an important risk factor for palpable and anaplastic tumors but not

for incidentally discovered tumors or well-differentiated cancers. Another prediagnostic study carried out later, in a group of 20,305 men in Maryland, by Braun *et al*. [48] could not confirm these results. A study by Gann *et al*. [49] also did not find a correlation between levels of circulating vitamin D metabolites and subsequent development of PCa. However in a further analysis of their data, Corder *et al*. [50] subsequently found that $1,25(OH)_2D$ levels showed a seasonal variation in PCa cases but not in controls, with a nadir in summer months. This may explain the lack of an effect in the Braun study due to the fact that serum collections occurred mainly in the fall, and as a result might have missed a nadir in levels among individuals that went on to develop PCa. A nested case-control study in Japanese-American men conducted in Hawaii [51] also did not find a strong association between serum $1,25(OH)_2D$ levels and the incidence of PCa, possibly due to the lack of sufficient number of study subjects with low vitamin D levels.

3. VITAMIN D BINDING PROTEIN

The vitamin D binding protein (DBP) may modulate vitamin D action by controlling the levels of free 25(OH)D or $1,25(OH)_2D$ available to activate the VDR (see Chapter 8). As such, it may play a role in the etiology of PCa. However, studies in this area have produced divergent results. Corder *et al*. [50] failed to find an association with DBP and PCa in their cohort of subjects. In a group of 68 men with PCa, Schwartz *et al*. [52] found that DBP levels were significantly higher in individuals with PCa compared to controls. Individuals with DBP levels >350 mg/L had a greater than fivefold increase in PCa risk.

D. Genetic Factors

1. VDR POLYMORPHISMS AND PCA RISK

As in other target tissues, the mechanism of action of $1,25(OH)_2D$ in the prostate involves hormone action through the classical ligand-dependent activation of genes via the vitamin D receptor (VDR) (see Chapters 11,13). Several polymorphisms have been identified in the VDR gene that may contribute to the risk of osteoporosis [53] (see also Chapter 68). Some of these polymorphisms may contribute to PCa risk as well [54]. Ingles *et al*. [55] studied the VDR polymorphism due to an increased number of adenosine residues in a microsatellite poly-A tract in the noncoding region of exon 9. They found that the presence of the long (L) VDR allele (with poly-A of >18) versus the short allele (S with poly-A <18), whether in the homozygous state (LL) or heterozygous state (LS), was associated with a four- to fivefold increase in PCa risk. This polymorphism is linked to

the more commonly studied VDR polymorphisms at the BsmI and TaqI sites in intron 8 and exon 9 of the VDR gene. Taylor *et al*. [56] examined the association between the TaqI polymorphism and PCa risk. Their results showed that men homozygous for the *t* allele (presence of the TaqI site) had one-third less risk of developing PCa requiring prostatectomy [56]. Based on linkage disequilibrium between the T allele and the L allele, it appears that these VDR polymorphisms are associated with an increased risk of PCa. However, a recent study of the TaqI polymorphism in 400 patients with BPH who have been followed clinically for a median of 11 years did not find an association between the risk of developing PCa and the TaqI variant genotype [57]. Several studies also report a lack of association between PCa risk and the TaqI polymorphism or poly-A tract length [58–61]. Some recent reports show that the presence of the B genotype (lack of BsmI site) in the homozygous (BB) or heterozygous (Bb) state lowers the risk of PCa when compared to the bb genotype [62,63]. However, Suzuki *et al*. [64] could not confirm any significant association between the BsmI, ApaI, and TaqI VDR polymorphisms and familial PCa risk in a Japanese population. The VDR polymorphisms discussed so far do not alter the amino acid sequence of the VDR protein. However, it is possible that they could alter VDR mRNA expression or stability and thereby affect the abundance of VDR.

The FokI polymorphism at the start codon of the VDR gene results in amino acid changes in the VDR protein. The ATG variant (f allele or M_1 containing the FokI site) initiates at this site and codes for a VDR three amino acids longer than the ACG variant (F allele or M_3 without the FokI site) that initiates at the second ATG, 3 amino acids downstream [65]. Xu *et al*. [66] examined the association of the FokI genotype with the histopathological characteristics and prognosis of PCa among cancer patients who had undergone radical prostatectomy. They found that subjects with the ff genotype had a significantly lower mean percentage of Gleason grade 4/5 cancer and concluded that the ff genotype was associated with less aggressive histopathological findings than the Ff or FF genotypes [66]. Interestingly, regarding the FokI polymorphism, it appears that the alleles that may be protective against PCa in men may be predictive of low bone mass in some groups of women (see Chapter 68).

In a population of PCa patients from Shanghai, Chokkalingam *et al*. [67] did not find an association between the FokI polymorphism and PCa risk. Interestingly, however, these investigators found that in men with the ff genotype, those in the highest tertile of plasma insulin-like growth factor binding protein-3 (IGFBP-3) had a decreased risk of PCa versus those in the lowest tertile. These results suggest that the IGF/IGFBP axis and vitamin D regulatory systems may interact to affect PCa risk. The clinical utility of these VDR polymorphisms in predicting PCa risk is not entirely clear and the subject requires further investigation. Haussler and colleagues [68] have suggested that the F and f alleles have differential ability to transactivate target genes explaining why the FokI site might alter disease risk (see Chapters 13 and 68). This data may rationalize why the F variant may be protective in osteoporosis in women but it is counterintuitive for the increased risk or worse prognosis of prostate cancer in men associated with the F genotype.

II. PROSTATE AS A TARGET FOR VITAMIN D

A. Vitamin D Is an Antiproliferative and Prodifferentiation Agent

Although the role of vitamin D in maintaining calcium homeostasis has been understood for a long while (see Chapter 24), it is only recently that investigators have begun to understand the broader scope of vitamin D actions [69]. In addition to exhibiting immunomodulatory effects (see Chapter 36), 1,25-$(OH)_2D$ has been shown to have antiproliferative and prodifferentiating actions in a number of tumors and malignant cells including PCa [29–34,70], raising the possibility of its use as an anti-cancer agent.

The epidemiological evidence described above supports the notion that the prostate represents a vitamin D target organ. The finding by Miller *et al*. [71] of the presence of VDR in LNCaP human PCa cells and the demonstration of VDR and its antiproliferative actions in three different PCa cell lines including LNCaP, PC-3, and DU 145 cells by Skowronski *et al*. [72] were important early findings that suggested that 1,25$(OH)_2D$ might play a direct role in prostate biology.

B. VDR in the Normal Prostate

Although the initial description of VDR in prostate and most of the subsequent investigation has centered around PCa cell lines, 1,25$(OH)_2D$ also appears to play an important role in normal prostate tissue. Peehl *et al*. [73] reported the presence of VDR in freshly obtained surgical prostate specimens as well as primary cultures of epithelial and stromal cells of the prostate. Primary cultures from surgical specimens of BPH also demonstrated VDR [73]. Although VDR were present in both epithelial and stromal cells cultured separately, lower

levels of VDR were seen in the stromal fibroblasts compared to cells of the glandular epithelium. The region of origin within the prostate tissue did not influence the abundance of VDR, as both the peripheral zone and central zone cultures had similar amounts of VDR [73]. The presence of VDR has also been demonstrated in the secretory epithelium and stromal cells from human prostate tissue [74] and in human neonatal prostatic epithelial cells transformed with Simian Virus 40 (SV40) [75]. Krill *et al*. [76] studied VDR expression in normal prostate glands from donors of various age groups and found that VDR expression changed with age with peak levels in the fifth decade and a decline thereafter. Vitamin D has been shown to exert antiproliferative effects on rat neonatal prostatic epithelial cells [77] and human prostatic epithelial cells [73,78]. Konety *et al*. [77,79] showed that in the rat, exposure of pups *in utero* to administered $1,25(OH)_2D_3$ influenced prostatic growth and differentiation throughout the life of the animal. In general, all of these studies support a role of vitamin D in normal prostate physiology and growth.

FIGURE 1   Dose response effect of $1,25(OH)_2D_3$ on the proliferation of human prostate cancer cell lines. LNCaP (circles), PC-3 (triangles) and DU 145 (squares) cells seeded in 6-well plates were treated for 6 days with vehicle or indicated concentrations of $1,25(OH)_2D_3$ in culture media. Proliferation was assessed by the determination of DNA content at the end of 6 days. Values shown are mean ± SD from two to four experiments done in duplicate. From Skowronski *et al*. [72] with permission.

III. INHIBITION OF PROSTATE CANCER GROWTH BY VITAMIN D

A. *In Vitro* Studies in Prostate Cells

1. CANCER CELL LINES

Miller and co-workers [71] demonstrated the presence of VDR in LNCaP human PCa cells. In their study, $1,25(OH)_2D_3$ at concentrations from 10^{-11}–10^{-9} M was slightly stimulatory to cell growth, when the cells were cultured in media supplemented with charcoal-stripped serum depleted of endogenous androgens, conditions under which LNCaP cells grew very poorly. A subsequent study by Skowronski *et al*. [72] demonstrated the presence of VDR in LNCaP cells as well as two other human prostate cancer cell lines DU 145 and PC-3. Interestingly, Skowronski and co-workers found $1,25(OH)_2D_3$ to exert growth-inhibitory effects upon these cell lines (Fig. 1). The growth inhibition in LNCaP cells was quite striking (~ 60%) when cultured in the presence of increasing concentrations of $1,25(OH)_2D_3$ up to 100 nM in regular growth medium containing 5% fetal bovine serum (FBS), a medium supporting robust cell growth. The discrepancy between the effect of $1,25(OH)_2D_3$ on the growth of LNCaP cells reported in these studies may be due to differences in the culture conditions. Indeed, as shown in a later study by Zhao *et al*. [80], androgens present in serum in the growth medium influenced the effect of vitamin D on LNCaP cell growth (see Section III.C below). Esquenet *et al*. [81]
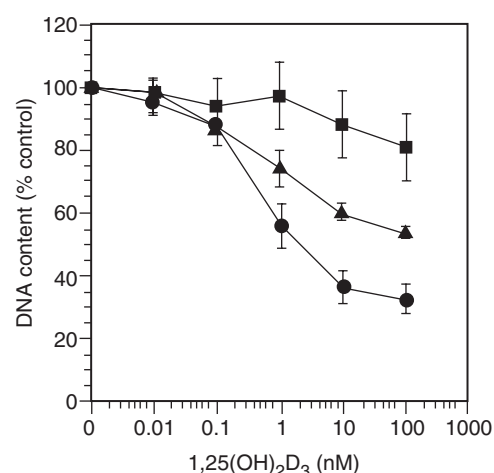
also reported significant inhibition of LNCaP cell growth by $1,25(OH)_2D_3$ in the presence of androgens.

The study by Skowronski *et al*. [72] demonstrated growth inhibition by vitamin D in other PCa cells such as PC-3 and DU 145 (Fig. 1). The magnitude of growth inhibition was less in PC-3 cells (~40–50%) when compared to LNCaP cells, and the growth inhibition seen in DU 145 cells was minimal. Interestingly, Skowronski *et al*. also reported an inverse correlation between $1,25(OH)_2D_3$ induction of 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase) and $1,25(OH)_2D_3$-mediated growth inhibition in these cells. They found that 24-hydroxylase mRNA was induced by $1,25(OH)_2D_3$ maximally in DU 145 and substantially in PC-3 cells while the induction of 24-hydroxylase mRNA was not detected in LNCaP cells (see Section III.B below). So there is limited correlation between growth inhibition and induction of specific genes. $1,25(OH)_2D_3$ has also been shown to inhibit the growth of other prostate cancer cell lines such as ALVA 31, PPC-1 [82], and MDA PCa 2a and 2b cells [83].

2. PRIMARY PROSTATE CELLS

The use of primary cultures of prostate cancer cells provides an important tool to study cancer biology as they may be more closely related to the clinical setting than established cancer cell lines. Peehl *et al*. [73] reported the results of their investigation of a series of primary prostate cancer cell cultures. Epithelial cultures were generated from surgical specimens obtained
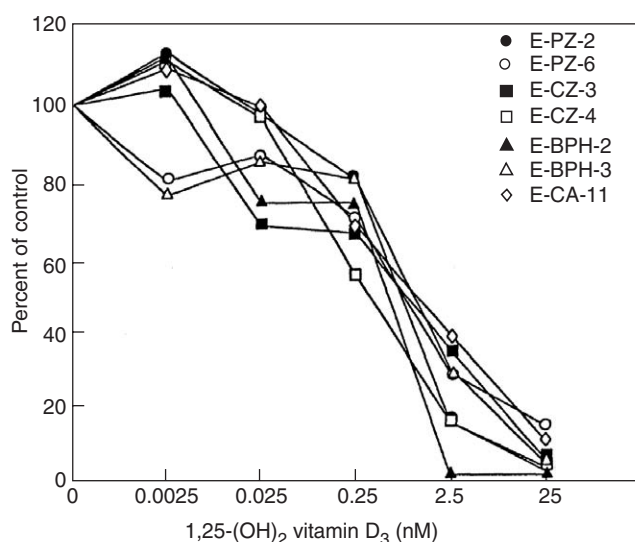
FIGURE 2 $1,25(OH)_2D_3$ causes growth inhibition in normal and malignant primary prostatic epithelial cells. Proliferation was determined by clonogenic assays in serum-free medium after 10 days of treatment with increasing doses of $1,25(OH)_2D_3$ as indicated. E-PZ and E-CZ represent normal prostatic epithelial cell lines. E-BPH-2 and -3 are epithelial cell cultures derived from benign prostatic hyperplasia specimens. E-CA-11 refers to an epithelial cell culture generated from prostatic carcinoma. From Peehl et al. [73] with permission.

during prostatectomy. $1,25(OH)_2D_3$ ligand-binding experiments revealed variable levels of VDR abundance among the different cultures. The VDR abundance did not correlate with histological grade of the tumors. As shown in Fig. 2, in clonogenic growth assays, $1,25(OH)_2D_3$ exhibited a substantial inhibition ($ED_{50} = 0.25 - 1$ nM) of the growth of these primary epithelial cells derived from the peripheral and central zones of normal prostate as well as from BPH and cancer. Interestingly, the growth inhibition was irreversible after withdrawal of $1,25(OH)_2D_3$ from the medium [73]. In contrast, the growth inhibition seen in LNCaP cells is reversible [81].

Peehl et al. [73] also studied cultures of stromal fibroblasts generated from the prostate surgical specimens and found that these cells also contained VDR and were inhibited by $1,25(OH)_2D_3$. The abundance of VDR was less in the stromal fibroblasts, and the growth inhibition by $1,25(OH)_2D_3$ was less when compared to the epithelial cultures. The significance of the presence of VDR in the fibroblastic component of the prostate is uncertain. However, there is evidence that fibroblasts may influence prostate epithelial cell growth through the production of growth factors that act in a paracrine manner [84,85]. $1,25(OH)_2D_3$ may potentially regulate stromal production of some of these growth factors and thereby control the growth of prostatic epithelium [86,87].

3. VIRALLY TRANSFORMED PROSTATE CELLS

The large T antigen of simian virus 40 (SV40) and the E6 and E7 proteins of human papilloma virus (HPV) cause disruptions in the p53 and retinoblastoma (Rb) genes [88,89] and have been used to transform prostate cells into immortalized cell lines [90,91]. Studies of such virally transformed prostate cell lines are useful models to help gain an understanding of the role of p53 and Rb genes in the pathogenesis of prostate cancer and to potentially elucidate the mechanism of the growth-inhibitory action of $1,25(OH)_2D_3$. Investigators have evaluated VDR and $1,25(OH)_2D_3$ action in both SV40- and HPV-transformed cell lines [90,92,93]. These studies showed that high affinity VDRs were present in the SV40 and HPV transformed cell lines and that 24-hydroxylase mRNA was induced by $1,25(OH)_2D_3$ treatment of these virally transformed cell lines. However, the effect of $1,25(OH)_2D_3$ on growth was different in the SV40- and HPV-transformed cell lines. Whereas the growth of HPV-transformed cell lines was inhibited by $1,25(OH)_2D_3$, the SV40-transformed cell lines were resistant to $1,25(OH)_2D_3$ action in terms of an antiproliferative response. As discussed in the section below, the expression of the large T antigen appears to cause resistance to $1,25(OH)_2D_3$ action on proliferation in prostate as well as breast epithelial cells due to an inhibition of the transcriptional activity of VDR [94].

4. RESISTANCE OF SOME PROSTATE CELLS TO GROWTH INHIBITION BY VITAMIN D

While many of the established PCa cell lines or primary cultures of adenocarcinoma-derived cells respond to vitamin D, there is evidence that some PCa cells may become resistant to antiproliferative activity of $1,25(OH)_2D_3$. This resistance can develop at several levels: (i) through the loss or decreased expression of VDR or the retinoid X receptor (RXR), (ii) through VDR polymorphisms that diminish its function, (iii) through elevated expression of VDR co-repressors, (iv) by increased expression of enzymes that metabolize $1,25(OH)_2D_3$, or (v) by other means. An example of a loss of response to vitamin D due to the loss of VDR is seen in the cancer cell line JCA-1 which does not respond to $1,25(OH)_2D_3$ with growth inhibition [95]. VDR is not detectable in these cells and stable transfection of the cells with a VDR cDNA makes these cells sensitive to growth inhibition by vitamin D [95].

As discussed above, transformation of prostatic epithelial cells by SV40 but not HPV results in a loss of growth inhibitory effect of $1,25(OH)_2D_3$ in the transformed cells. Human breast epithelial cells transformed by the SV40 large T antigen have also been shown to become resistant to $1,25(OH)_2D_3$ [94], demonstrating

the generality of this finding. The authors of this study showed that the expression of the large T antigen strongly inhibited $1,25(OH)_2D_3$–induced VDRE transcriptional activity in a dose-dependent manner and that increasing the VDR concentration could reverse the inhibitory effect of the large T antigen. Indeed, the resistance to $1,25(OH)_2D_3$–mediated growth inhibition seen in the breast epithelial cells could be overcome by the overexpression of VDR in the cells [94].

As shown previously [72], $1,25(OH)_2D_3$ did not inhibit the growth of DU 145 cells despite the presence of a functional VDR in these cells. DU 145 cells, like JCA-1 and PC-3 cells, have relatively low numbers of VDR, and stable transfection of VDR into these cells somewhat restored the growth inhibitory activity of $1,25(OH)_2D_3$ [96]. However, DU 145 cells also have the highest levels of $1,25(OH)_2D_3$–inducible 24-hydroxylase activity among the different prostate cancer cell lines studied so far. 24-Hydroxylase initiates the catabolism of $1,25(OH)_2D_3$ to inactive metabolites. As discussed in detail in the following section (Section III.B), adding an inhibitor of 24-hydroxylase resulted in a significant inhibition of DU 145 cell growth by $1,25(OH)_2D_3$ [97].

One important concept that has emerged from these studies is that different signaling pathways are used by VDR to regulate genes involved in growth control versus those involved in other functions. This is apparent in the ability of cells resistant to growth inhibition by $1,25(OH)_2D_3$ to still exhibit VDR up-regulation [97] and/or 24-hydroxylase induction in response to $1,25(OH)_2D_3$ [72,97].

B. Cellular Responsiveness to $1,25(OH)_2D_3$—Role of Enzymes Involved in Vitamin D Metabolism

The key enzymes involved in vitamin D metabolism are 24-hydroxylase (CYP24), which catalyzes the initial step in the conversion of $1,25(OH)_2D_3$ to less active metabolites (see Chapter 6) and 1α-hydroxylase (CYP 27B1), which catalyzes the synthesis of $1,25(OH)_2D_3$ from $25(OH)_2D_3$ (see Chapter 5). As discussed in the following sections the level of expression of these enzymes in target cells such as PCa cells influences the magnitude of the growth-inhibitory responses to vitamin D metabolites.

1. 25-Hydroxyvitamin D₃ 24-Hydroxylase (CYP24)

$1,25(OH)_2D_3$ induces the expression of the enzyme 25-hydroxyvitamin D_3 24-hydroxylase (24-hydroxylase) in target cells, which catalyzes the initial step in the conversion of the active molecule $1,25(OH)_2D_3$ into less active metabolites. An interesting study by

Albertson *et al*. [98] used comparative genomic hybridization (CGH) to resolve two regions of amplification within an approximately 2 Mb region of recurrent aberration at 20 q13.2 in breast cancer. A known putative oncogene ZNF217 mapped to one peak, and CYP24 (encoding vitamin D 24-hydroxylase), whose overexpression likely leads to the abrogation of growth control mediated by vitamin D, mapped to the other, raising the possibility that the CYP24 gene encoding 24-hydroxylase might be an oncogene. It is clear that in normal as well as malignant cells induction of 24-hydroxylase by $1,25(OH)_2D_3$ would limit its own growth-inhibitory actions.

In prostate cells, the degree of growth inhibition of vitamin D has been shown to be inversely proportional to the 24-hydroxylase activity in the cells. Among the human PCa cell lines DU 145, PC-3, and LNCaP, DU 145 cells exhibit the highest level of 24-hydroxylase induction and are the least responsive to $1,25(OH)_2D_3$ in terms of growth inhibition [72,82]. On the other hand, the basal and induced expression of 24-hydroxylase is very low in LNCaP cells, and growth inhibition by $1,25(OH)_2D_3$ is substantial. Ly *et al*. [97] examined the possibility that inhibition of 24-hydroxylase activity would render DU 145 cells more sensitive to the antiproliferative effect of $1,25(OH)_2D_3$. The results of their investigation show that in DU 145 cells, liarozole (an imidazole derivative that inhibits P450 hydroxylases) causes significant inhibition of 24-hydroxylase activity leading to an increase in $1,25(OH)_2D_3$ half-life in DU 145 cells and thereby allows a substantial antiproliferative effect [97].

In many target cells, $1,25(OH)_2D_3$ has been known to cause homologous up-regulation of its receptor levels [99,100]. Prolongation of $1,25(OH)_2D_3$ half-life (due to the inhibition of 24-hydroxylase by liarozole) therefore resulted in an enhanced up-regulation of VDR in DU 145 cells, making them more responsive to $1,25(OH)_2D_3$. As shown in Fig. 3, the growth inhibition due to the treatment of DU 145 cells with $1,25(OH)_2D_3$ alone or liarozole alone was minimal while the combination of both these agents resulted in a substantial inhibition of cell growth. This increase in growth inhibition could be due to both an extension of the $1,25(OH)_2D_3$ half-life and enhanced VDR up-regulation resulting from the inhibition of 24-hydroxylase by liarozole. Miller *et al*. [82] also demonstrated that the differences in $1,25(OH)_2D_3$–mediated growth inhibition between various PCa cell lines correlate inversely to 24-hydroxylase expression in these cells. A recent study by Peehl *et al*. [101] has shown that in primary human PCa cells, the use of the P450 inhibitor ketoconazole potentiates the growth inhibitory effects of $1,25(OH)_2D_3$ or its structural analog EB 1089 by

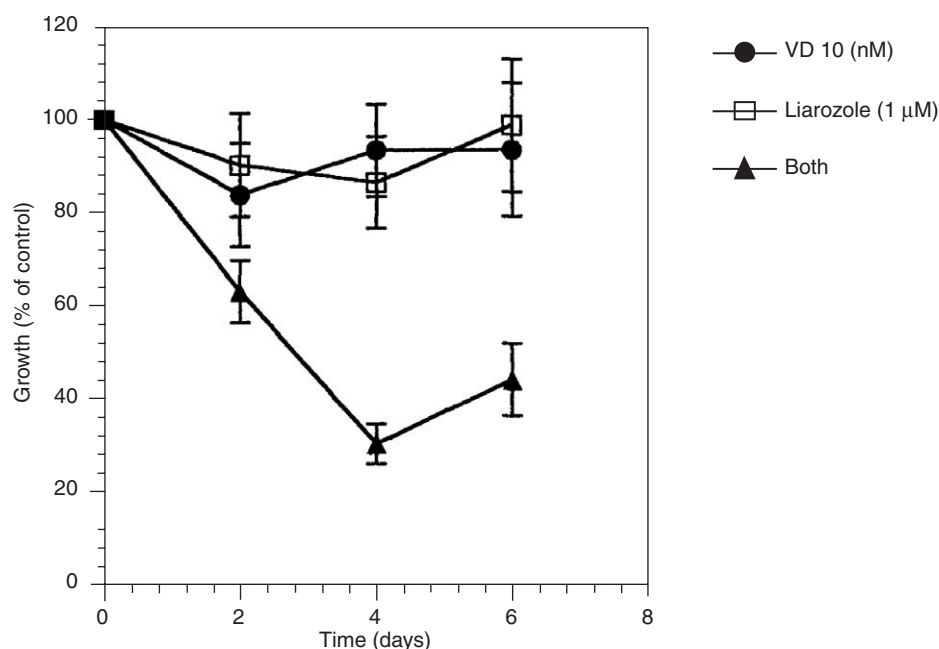FIGURE 3   Effect of $1,25(OH)_2D_3$ and liarozole on DU 145 cell growth. Effect of $1,25(OH)_2D_3$ (10 nM), liarozole (1 μM), or a combination of both on DU 145 cell growth was determined over a time course of 6 days with fresh medium containing the agents being replenished every 2 days. Cell proliferation was estimated using the MTI assay. Data are expressed as mean ± SD of three experiments. *$p < 0.05$ compared with vehicle treated control group. From Ly *et al.* [97] with permission.

inhibiting the 24-hydroxylase activity in these cells. Thus, combinations of $1,25(OH)_2D_3$ with inhibitors of 24-hydroxylase such as ketoconazole or liarozole may enhance its anti-tumor effects in PCa therapy. The combination approach may also allow the use of $1,25(OH)_2D_3$ at lower concentrations thereby reducing the hypercalcemic side effects.

2. 25-HYDROXYVITAMIN D₃ 1α-HYDROXYLASE (CYP27B1)

The active hormone $1,25(OH)_2D_3$ is formed in the kidney by the hydroxylation of $25(OH)D_3$ at the C-1 position by the enzyme 1α-hydroxylase (see Chapter 5). The kidneys are the major source of circulating $1,25(OH)_2D_3$ in the body. In recent years, however, the presence of extra-renal 1α-hydroxylase has been demonstrated, which contributes to the local production of $1,25(OH)_2D_3$ within various tissues (see Chapter 79). Schwartz *et al.* [102] showed that normal human prostatic epithelial cells express 1α-hydroxylase. They raised the possibility that treatment with $25(OH)D_3$ could potentially inhibit the growth of PCa, due to local production of $1,25(OH)_2D_3$ within the prostate, thus avoiding the systemic side effect of hypercalcemia due to $1,25(OH)_2D_3$ administration. The ability of $25(OH)D_3$ to cause hypercalcemia is much reduced because of its lower affinity for the

VDR. Results of a study by Barreto *et al.* [103] support this hypothesis by demonstrating the growth inhibitory effect of $25(OH)D_3$ in primary epithelial cell strains derived from normal human prostatic peripheral zone.

A recent study by Hsu *et al.* [104] quantitated the levels of 1α-hydroxylase in primary prostatic epithelial cells derived from normal tissue, BPH, or cancer as well as in established PCa cell lines. This study shows that epithelial cells from normal prostate have more 1α-hydroxylase activity than those derived from BPH or cancer. The activity in primary cancer cells is lower than BPH, and the PCa cell lines express the lowest 1α-hydroxylase activity. Whitlatch *et al.* [105] similarly found reduced 1α-hydroxylase activity in prostate cancer cells compared to normal prostatic cells. The decrease in 1α-hydroxylase enzyme activity in PCa cells may arise from a decrease in 1α-hydroxylase gene promoter activity in these cells [106]. Segersten *et al.* [107] examined 1α-hydroxylase expression by RT-PCR and immunohistochemical analyses and reported that the expression of 1α-hydroxylase is lower in parathyroid carcinomas, compared with normal parathyroid tissue. However, studies on tissues derived from normal colon and colon carcinoma show elevated levels of 1α-hydroxylase in colon carcinoma [108]. The Hsu *et al.* study [104] also shows that the antiproliferative effect of $25(OH)D_3$ correlates
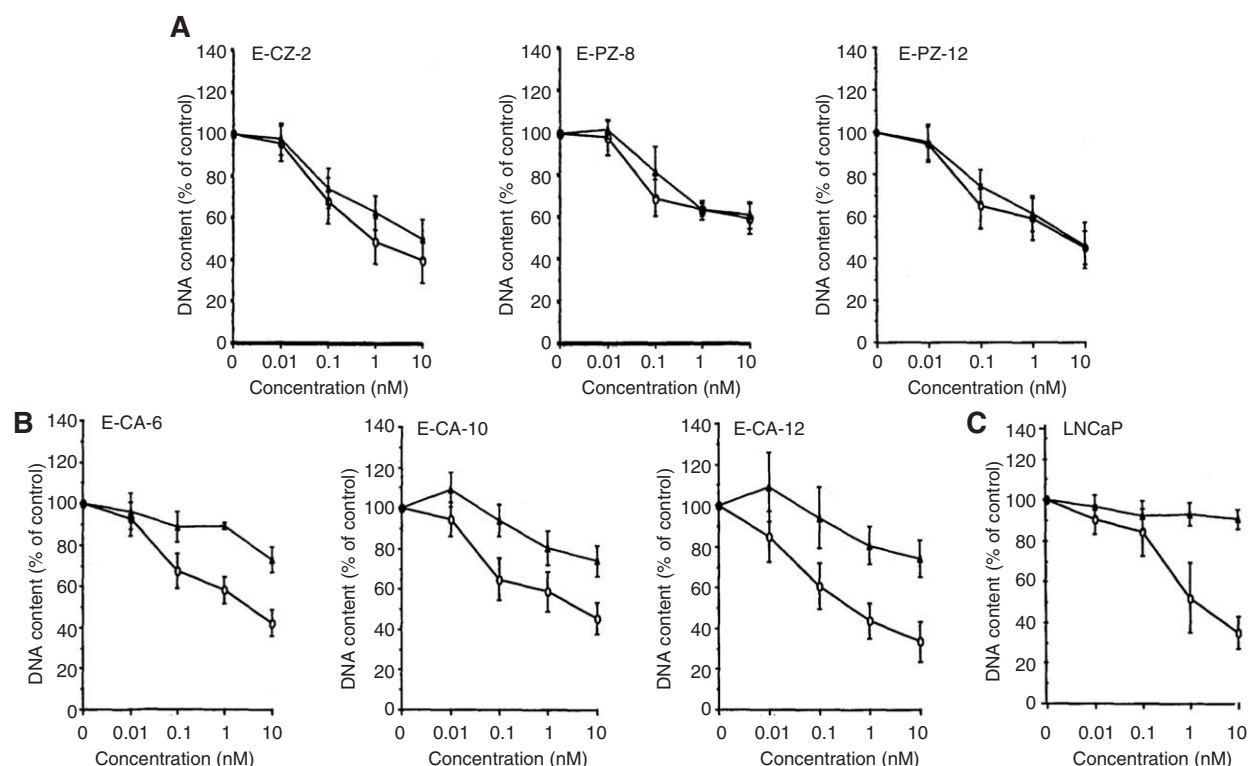
FIGURE 4   Growth inhibition by 25(OH)D$_3$ (solid triangles) in comparison with 1,25(OH)$_2$D$_3$ (open circles) in normal and malignant prostate epithelial cells. Cells were plated and treated with various concentrations of the vitamin D metabolite for 6 days and DNA contents were assayed. **A**. In normal prostatic epithelial cells (E-CZ-2, E-PZ-8, and E-PZ-12), 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ were equally growth inhibitory at concentrations between 0.01 and 10 nM. **B**. In primary cultures of cancer cells (E-CA-6, E-CA-10, E-CA-12), 1,25(OH)$_2$D$_3$ induced ~20–40% more growth inhibition than 25(OH)D$_3$. **C**. The prostate cancer cell line LNCaP, although fully responsive to 1,25(OH)$_2$D$_3$, was resistant to treatment with 25(OH)D$_3$. From Hsu *et al*. [104] with permission.

with the endogenous 1α-hydroxylase activity in prostate cells. As illustrated in Fig. 4, the growth of primary epithelial cells from normal tissue or BPH is inhibited by 25(OH)D$_3$ to an extent similar to 1,25(OH)$_2$D$_3$, as it could be converted to 1,25(OH)$_2$D$_3$ by endogenous 1α-hydroxylase activity. In contrast, in primary epithelial cells from cancer or in the LNCaP human PCa cell line, with very low endogenous 1α-hydroxylase activity, the antiproliferative action of 25(OH)D$_3$ is much less pronounced in comparison to 1,25(OH)$_2$D$_3$. Importantly, the findings of reduced 1α-hydroxylase in cancer-derived prostatic epithelial cells raises the possibility that this difference may endow the malignant cells with an intrinsic growth advantage because of the resultant decrease in the local production of the growth inhibitory agent 1,25(OH)$_2$D$_3$. In addition, local deficiency of 1,25(OH)$_2$D$_3$ may allow cellular de-differentiation and invasion, hallmarks of malignancy.

A recent study by Hawkins *et al*. [109] examined three frequent single nucleotide polymorphisms present in the 1α-hydroxylase gene in PCa patients and control subjects and concluded that this gene does not play a major role in PCa susceptibility. The evidence

from *in vitro* studies, however, suggests that a decrease in 1α-hydroxylase activity may represent an important mechanism in PCa development and/or progression and that the administration of 25(OH)D$_3$ might be an effective chemopreventive approach while 1α-hydroxylase is initially still high within the prostate [104].

C. Vitamin D and Androgen Interactions

Androgens acting through the AR regulate prostate growth and play an important role in the development and progression of PCa [5]. *In vitro* studies have shown that there is cross talk between 1,25(OH)$_2$D$_3$ and androgen signaling in the androgen-responsive PCa cell line LNCaP [80,110]. 1,25(OH)$_2$D$_3$ up-regulates AR gene expression at both mRNA and protein levels and also increases PSA expression in LNCaP cells [111,112]. The secretion of PSA by LNCaP cells is synergistically enhanced when the cells are exposed to a combination of androgens and 1,25(OH)$_2$D$_3$, probably due, in part, to the up-regulation of AR by 1,25(OH)$_2$D$_3$ [110]. The antiproliferative action of 1,25(OH)$_2$D$_3$ in

LNCaP cells appears to be androgen-dependent as it could be blocked by the AR antagonist casodex [80].

Recent investigations involving cDNA microarray analyses of $1,25(OH)_2D_3$–regulated target genes in LNCaP cells reveal that several of the $1,25(OH)_2D_3$–regulated genes in these cells that modulate cell growth are also androgen responsive genes [113]. However, this androgen-dependent mechanism of $1,25(OH)_2D_3$ action may be specific to LNCaP cells because $1,25(OH)_2D_3$ also inhibits the growth of other PCa cells that do not express the AR [72,73]. Zhao et al. [83] have shown that $1,25(OH)_2D_3$ inhibits the growth and up-regulates AR expression in MDA PCa 2a and 2b cells that were recently established from a bone metastasis in a patient who exhibited advanced AIPC. In contrast to LNCaP, however, the growth-inhibitory action of $1,25(OH)_2D_3$ in the MDA PCa cells appears to be androgen-independent [83]. Yang et al. [114] studied the growth properties of a LNCaP derived androgen-independent subline, LNCaP-104R1, and found that these cells, unlike the parental LNCaP cells, grew well in medium containing charcoal-stripped serum depleted of endogenous androgens. Under these conditions $1,25(OH)_2D_3$ caused significant growth inhibition of these androgen-independent cells that was not abolished by the anti-androgen casodex [114]. Importantly these findings as well as the inhibition of AR negative cells [72,73,83,97] support the potential therapeutic role of vitamin D in the treatment of AIPC.

D. *In Vivo* Studies in Animal Models

Although several rodent models of PCa have been developed [115–118], there is still a lack of a perfect animal model for human PCa. Several researchers have developed human prostate tumor xenograft models by transplanting clinical prostate tumors or cultured human PCa cells into immune-deficient mice [119]. Using these animal models investigators have attempted to support the *in vitro* studies showing inhibition of PCa cells by vitamin D [29–34,70].

As discussed in detail in several sections of this book, the concentrations of $1,25(OH)_2D_3$ required to produce a significant antiproliferative effect *in vivo* causes hypercalcemia as a side effect. Therefore, investigators have used structural analogs of $1,25(OH)_2D_3$ that exhibit reduced hypercalcemic effects in several *in vivo* animal studies as well as in clinical trials. Schwartz et al. [120] showed that administration of the vitamin D analog 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 (Ro23-7553) to mice bearing PC-3 xenografts resulted in a 15% decrease in tumor volume without significant increases in serum calcium levels.
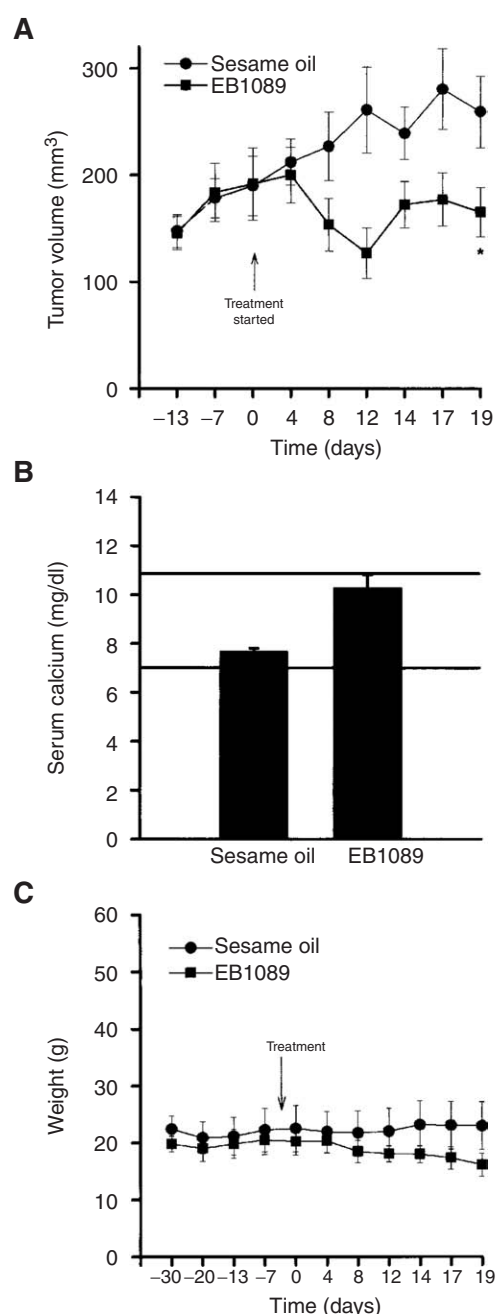
FIGURE 5   EB 1089 inhibits the growth of LNCaP xenografts in nude mice. One million LNCaP cells were mixed with 100 μl of Matrigel Matrix and injected subcutaneously into one site on the dorsal side of male nude mice (approximately 6 weeks of age). Tumors were allowed to grow to a volume of 150 mm³. Vehicle (sesame oil) or 0.5 μg/kg of EB 1089 were injected intraperitoneally into the mice every other day. Points are an average of treatment groups (*n* = 10) and bars are mean ± SE. **Panel A**. Tumor volume. **Panel B**. Average serum calcium at the end of the study. **Panel C**. Average body weight in control and treatment groups. From Blutt et al. [121] with permission.

Blutt *et al.* [121] showed that intraperitoneal injections of the vitamin D analog EB 1089 reduced growth of LNCaP tumors in a nude mouse xenograft model. As illustrated in Fig. 5, intraperitoneal injections of EB 1089 into athymic male *nu/nu* mice bearing LNCaP xenografts substantially reduced tumor volume (Fig. 5A) without raising serum calcium levels beyond the normal range (Fig. 5B) or causing weight loss (Fig. 5C). These data suggest that EB1089 is a promising candidate for PCa treatment. A recent study by Koeffler and colleagues reported inhibition of LNCaP xenograft growth in nude mice by three different vitamin D analogs without increases in serum calcium levels [122].

Other models of PCa have been developed in rats, mice, and dogs (reviewed in [118]), in addition to the xenografts of human PCa tumor tissue or cells in immune-compromised hosts. One of the first established and widely used models is the Dunning rat model [123]. The original Dunning R-3327 was a spontaneous rat prostatic adenocarcinoma and a variety of sublines have been developed from it including the highly metastatic MAT LyLu subline which when injected into rats forms highly metastatic tumors [124]. Getzenberg *et al.* [115] demonstrated that $1,25(OH)_2D_3$ and the analog Ro23-6760 inhibited the growth of the MAT LyLu tumors in rats. Another study of the MAT LyLu tumor model in rats showed that $1,25(OH)_2D_3$ and the analog EB 1089 decreased tumor size and the number of lung metastasis in these animals [125]. EB 1089 was significantly less hypercalcemic than $1,25(OH)_2D_3$ and did not induce severe weight loss [125]. Oades *et al.* [126] compared the action of $1,25(OH)_2D_3$ and the less hypercalcemic analogs EB1089 and CB1093 in three animal models of prostate cancer, MAT LyLu Dunning prostate model, PAIII tumors in Lobund-Wistar rats, and LNCaP xenografts in nude mice. Although both analogs increased serum calcium levels, the levels were significantly less than in rats treated with $1,25(OH)_2D_3$. Tumor growth was inhibited in male athymic nu/nu mice with LNCaP tumor xenografts. PAIII cells failed to express functional VDR and were insensitive to $1,25(OH)_2D_3$ and its analogs, either *in vitro* or *in vivo*. VDR-dependent growth inhibition and not the inhibition of angiogenesis was the main mechanism of action of these compounds *in vivo* [126]. Cumulatively, the results support the notion that the less hypercalcemic analogs of $1,25(OH)_2D_3$ offer a novel therapeutic option for treating prostate cancer.

Investigators have attempted to explore the chemopreventive activity, if any, of vitamin D compounds in animal models. Transgenic models of PCa have also been developed in mice (reviewed in [118]). In the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model, the prostate-specific rat probasin promoter has been used to drive the expression of the SV 40 large–T antigen coding region, and mice expressing the transgene display progressive forms of prostatic disease that histologically resemble human PCa [116]. Another model used to test the chemopreventive effect of vitamin D was the G gamma/T-15 transgenic mouse model of AIPC. Although the administration of the vitamin D analog EB 1089 did not alter the onset of tumors in these mice, it slowed down the rate of tumor growth [127]. Xue *et al.* [128] fed rats a high fat, low calcium and low vitamin D diet, which resulted in the hyperproliferation of the dorsal prostate epithelium which could promote tumorigenesis. Increasing the levels of calcium and vitamin D in the diet inhibited hyperproliferation, providing evidence for the anti-tumor activity of calcium and/or vitamin D in the diet.

Some studies have used $1,25(OH)_2D_3$ or its analogs in combination with other therapeutic agents and demonstrated their anti-tumor activity *in vivo* (for a detailed discussion of the combination approach, see Section VI). Thus *in vivo* models provide a valuable tool to demonstrate the anti-tumor activity of vitamin D compounds while monitoring their tendency to elevate serum calcium levels and thus validate their use in preparation for clinical trials.

IV. VITAMIN D ANALOGS

A. Potency Versus Toxicity

The concentrations of $1,25(OH)_2D_3$ used to produce significant antiproliferative effects in cultured PCa cells are often much higher than circulating levels of $1,25(OH)_2D_3$ and whether they can be safely achieved *in vivo* or not is being investigated (see Chapter 97). Use of high doses of $1,25(OH)_2D_3$ as a treatment for PCa and other cancers predictably results in hypercalcemia and hypercalciuria. This is the only side-effect that has been encountered, but potential hypercalcemia and renal stone formation limit the concentration of $1,25(OH)_2D_3$ that can be administered to patients. Consequently, structural analogs of $1,25(OH)_2D_3$ that effectively activate the VDR but are less hypercalcemic are being developed and evaluated for their potency as antiproliferative agents *in vitro* and *in vivo*. Section VIII of this book (Chapters 80–88) extensively reviews the currently available vitamin D analogs and the structure-function relationships that determine the relative hypercalcemic and differentiating potencies of these analogs. Several mechanisms could contribute to the differential activity of analogs on calcium metabolism and antiproliferation and are discussed in detail in Chapters 82 and 83. However, a complete understanding of the mechanistic basis for the separation

of hypercalcemic and antiproliferative activities has not yet been realized.

B. Vitamin D Analogs and Prostate Cancer

Many investigations have shown that a number of these vitamin D analogs can inhibit the growth of PCa cells in culture at concentrations lower than $1,25(OH)_2D_3$ [129–135]. Several of these analogs have also been shown to slow the growth of prostate xenograft tumors in animals (as discussed in Section III.E). For example, Schwartz et al. [129] showed that the 16-diene analogs were more effective in inhibiting the growth of PCa cells than $1,25(OH)_2D_3$ and unlike $1,25(OH)_2D_3$ the analogs inhibited the growth of DU 145 cells as well. A study by Skowronski et al. [130] demonstrated that the analogs EB 1089, MC903, 22-oxacalcitriol, and Ro24-2637 significantly inhibited the growth of PCa cells with ED_{50} values lower than for $1,25(OH)_2D_3$. The analogs were also more potent in stimulating the production of PSA regarded as a marker of differentiation in these cells. Similarly, 24-oxo metabolites of vitamin D [136] and fluorine derivatives of the $1,25(OH)_2D_3$ side-chain [131] also exhibited enhanced antiproliferative potencies. Hisatake et al. [137] report that a newly synthesized vitamin D analog $[1,25(OH)_2$-16-ene-5,6-trans-D_3 (Ro 25-4020)], that has a novel 5,6-trans motif, exhibits a 10- to 100-fold increase in antiproliferative activity in breast cancer and PCa cells while exhibiting at least 40-fold less hypercalcemic activity in mice. The higher activity than expected from its binding affinity for VDR is due, in part, to its metabolism to a 24-oxo metabolite, which retains significant biopotency [138]. Derivatives of vitamin D_2 that have been shown to be less hypercalcemic than $1,25(OH)_2D_3$ exhibit antiproliferative effects in PCa cells [139,140]. Interestingly, Polek et al. [141] showed that LG190119, one of a series of novel nonsteroidal VDR modulators, also inhibits LNCaP xenograft growth in mice without causing hypercalcemia. Nonsteroidal analogs are discussed in Chapter 88.

A recent report [142] showed that the vitamin D analog V (BXL-353) inhibited the growth of BPH cells in culture and stimulated apoptosis. When administered to intact or castrated rats supplemented with testosterone, BXL-353 reduced the androgen effect on ventral prostate weight without causing hypercalcemia or affecting sex hormone secretion, suggesting that it might be useful in the treatment of patients with BPH [142] (see also Chapter 104). Several of the vitamin D analogs have been tested in vivo and found to exhibit tumor inhibitory effects without inducing hypercalcemia (discussed in detail in Section III.E and

also see Fig. 5). It is hoped that some of these vitamin D analogs will emerge as clinically useful anti-cancer agents.

V. MECHANISMS OF VITAMIN D–MEDIATED GROWTH INHIBITION

Several studies have investigated the molecular mechanisms by which $1,25(OH)_2D_3$ or its analogs exert growth inhibitory effects on cancer cells including PCa cells. As described below, $1,25(OH)_2D_3$ seems to have multiple and diverse actions [143], often cell-specific, including effects on cell cycle arrest, apoptosis, inhibition of metastasis, and angiogenesis (see Chapters 89, 92, and 93). Investigators have also attempted to identify novel $1,25(OH)_2D_3$–target genes that mediate various actions of the hormone, especially the regulation of cell growth.

A. Growth Arrest

In many cancer cells, treatment with $1,25(OH)_2D_3$ or its analogs results in the accumulation of cells in the G_0/G_1 phase of the cell cycle [136], and this has been shown to be the case in LNCaP cells as well [136,144]. Treatment of LNCaP cells with $1,25(OH)_2D_3$ or the analog EB1089 caused an increase in the percentage of cells accumulating in the G_1 phase. The combination of 10^{-8} M $1,25(OH)_2D_3$ and 9-cis retinoic acid resulted in synergistic growth inhibition and caused more cells to accumulate in G_1 when compared to 10^{-8} M $1,25(OH)_2D_3$ alone.

There appear to be multiple mechanisms by which $1,25(OH)_2D_3$ causes cell cycle arrest. The retinoblastoma protein (Rb) is a key regulator of G_1 to S phase transition. Hyperphosphorylation of the Rb protein by G_1 cyclins and their cyclin-dependent protein kinase (CDK) partners inactivates the Rb protein and releases the repression on E2F transcriptional activity allowing cells to progress from G_1 to S phase. Zhuang and Burnstein [145] have shown that in LNCaP cells $1,25(OH)_2D_3$ exerts its effects on some of these key steps. $1,25(OH)_2D_3$ treatment of LNCaP cells causes an increase in the expression of the CDK inhibitor p21, a decrease in CDK2 activity leading to a decrease in the phosphorylation of Rb, and repression of E2F transcriptional activity resulting in G_1 arrest of the cells. Liu et al. [146] have shown that $1,25(OH)_2D_3$ directly up-regulates p21 gene expression in U937 leukemia cells, acting through a putative vitamin D response element (VDRE) in the promoter of the p21 gene. However, in LNCaP cells, the regulation of p21 gene

expression appears to be indirect [131,145]. Boyle *et al.* [147] showed that the induction of insulin-like growth factor binding protein-3 (IGFBP-3) gene expression in these cells by $1,25(OH)_2D_3$ results in increased p21 protein levels. The role of IGFBP-3 in mediating the growth inhibitory effect of $1,25(OH)_2D_3$ is discussed in detail in Section V.D. Moffatt *et al.* [148] showed that $1,25(OH)_2D_3$ inhibits growth and increases the expression of p21 mRNA and protein levels in ALVA-31 cells. $1,25(OH)_2D_3$–mediated growth inhibition of these cells is abolished by stable transfection of the cells with a p21 antisense construct demonstrating that p21 expression is necessary to mediate the antiproliferative effect of $1,25(OH)_2D_3$ in ALVA-31 cells.

A recent study by Polek *et al.* [149] investigated the role of p53 in $1,25(OH)_2D_3$–mediated cell cycle arrest through increases in p21 levels, as p21 is a known p53 target gene [150]. The findings of this study indicate that p53 is not required to induce growth inhibition or cause accumulation of cells in the G_1 phase by $1,25(OH)_2D_3$ in LNCaP cells. However, elimination of p53 function in LNCaP cells reduces G_0 arrest as measured by the loss of Ki67 expression, allows the cells to recover from $1,25(OH)_2D_3$-mediated growth arrest, and eliminates the growth inhibitory effects of combinations of 9-cis retinoic acid and $1,25(OH)_2D_3$ [149].

Although a functional Rb plays a key role in cell cycle control, lack of a functional Rb gene in DU 145 cells does not appear to be the critical reason for their reduced sensitivity to growth inhibition by $1,25(OH)_2D_3$. As detailed earlier in Section III.B, a combination of $1,25(OH)_2D_3$ and the 24-hydroxylase inhibitor, liarozole, causes appreciable growth inhibition in these cells [97]. Also, transfection of a functional Rb into DU 145 cells did not render the cells more sensitive to growth inhibition by $1,25(OH)_2D_3$ *in vitro* [93], even though the Rb-transfected DU 145 cells exhibited reduced tumorigenicity *in vivo* as xenografts in nude mice [151]. $1,25(OH)_2D_3$ does not increase p21 expression in PC-3 cells, which is consistent with the lack of G_1 accumulation of these cells following $1,25$-$(OH)_2D_3$ treatment. Thus, the regulation of cell cycle distribution by $1,25(OH)_2D_3$ appears to be cell-specific and may involve multiple pathways of action.

B. Apoptosis

Induction of apoptosis or programmed cell death by $1,25(OH)_2D_3$ is not uniformly seen in all cancer cells (see Chapter 93). In the case of PCa, investigators have mostly focused on LNCaP cells, and the findings have been variable. Zhuang and Burnstein [145] did not detect apoptosis in an adherent population of LNCaP

cells treated with 10 nM $1,25(OH)_2D_3$ using terminal transferase labeling. Hsieh and Wu [152] examined the nonadherent portion of a LNCaP cell population and found a small (35%) increase in hypodiploid cells following $1,25(OH)_2D_3$ treatment that was characteristic of apoptosis. Fife *et al.* [153] demonstrated DNA fragmentation in LNCaP cells treated with 10 nM $1,25(OH)_2D_3$ for four days. Blutt *et al.* [154] showed evidence of apoptosis in LNCaP cells treated with $1,25(OH)_2D_3$ for six days and also showed the downregulation of the pro-apoptotic proteins Bcl-2 and Bcl-X_L. They went on to demonstrate the involvement of Bcl-2 in $1,25(OH)_2D_3$–mediated apoptosis by stably transfecting the Bcl-2 gene into LNCaP cells and showing the loss of an apoptotic response to $1,25(OH)_2D_3$ in LNCaP cells that overexpressed Bcl-2. A more recent study by the same group [149] shows that p53 is not absolutely required for the induction of apoptosis in LNCaP cells by $1,25(OH)_2D_3$ and that the induction of apoptosis appears to be caspase-dependent. $1,25(OH)_2D_3$ down-regulates Bcl-2 expression even after the elimination of p53 function in LNCaP cells [149]. In LNCaP cells, therefore, $1,25(OH)_2D_3$ stimulates not only growth arrest but also apoptosis, although to a much lesser extent. Recent investigations report inhibition of anti-apoptotic proteins by vitamin D in other PCa cells. Guzey *et al.* [155] showed that in LNCaP and ALVA-31 cells, $1,25(OH)_2D_3$ decreased the expression of several anti-apoptotic proteins such as Bcl-2, Bcl-XL, and Mcl-1 leading to the activation of the mitochondrial pathway of apoptosis. The vitamin D analog V (BXL-353) has been shown to decrease Bcl-2 expression in DU 145 cells [156]. Induction of apoptosis by $1,25(OH)_2D_3$, however, appears to be cell-specific as it is not uniformly evident in all the cells that respond to $1,25(OH)_2D_3$ with growth inhibition. Even in LNCaP cells, where apoptosis has been demonstrated by some studies, the major action of $1,25(OH)_2D_3$ to inhibit cell growth appears to be cell cycle arrest [154].

C. Differentiation

$1,25(OH)_2D_3$ has been shown to induce the differentiation of a number of normal and malignant cells (see Chapter 92). However, as yet there is no strong evidence supporting a role for vitamin D as a differentiation-promoting agent in PCa. Peehl *et al.* [73] did not find any changes in cell morphology or in the expression of various keratins as markers of epithelial cell differentiation when primary human prostatic epithelial cells were exposed to $1,25(OH)_2D_3$. Konety *et al.* [157] harvested prostate tissue from castrated rats

treated with vehicle, testosterone (T), $1,25(OH)_2D_3$, or a combination of T and $1,25(OH)_2D_3$. Histological examination of the prostate tissue revealed a greater degree of epithelial cellular differentiation in rats treated with T and $1,25(OH)_2D_3$ compared to rats treated with T alone. In the PCa cells LNCaP and MDA PCa 2a and 2b, $1,25(OH)_2D_3$ increases the expression of PSA [83,110], which is regarded as a differentiation marker for prostatic epithelial cells. However, the effect appears to be much smaller compared to the induction of PSA expression by androgens, and the synergistic increase in PSA in cells treated with both $1,25(OH)_2D_3$ and androgens is in part due to the up-regulation of AR levels by $1,25(OH)_2D_3$ [83].

D. Growth Factor Actions

Growth factors play an important role in the regulation of prostate epithelial cell growth by autocrine and paracrine mechanisms. Prostatic stromal cells are capable of modifying the epithelial cell environment through paracrine production of peptide growth factors that can act on the basal epithelium [9,158]. The stromal cells are androgen-responsive [158], and the expression of VDR has also been demonstrated in the stromal fibroblasts, although at levels lower than the

epithelial cells [73]. Expression of autocrine growth factors by the epithelium may contribute to the progression of PCa through the development of independence from epithelial-stromal interactions that modulate the growth and development of the normal prostate gland. Some important growth factors that regulate prostate epithelial growth include epidermal growth factor, keratinocyte growth factor, basic fibroblast growth factor, transforming growth factors (TGFs), and insulin-like growth factors (IGFs). Some of these growth factors also appear to play an important role in the establishment and growth of metastatic PCa cells in bone [159,160]. The following is a discussion of the role played by members of two prominent growth factor families, namely IGFs and TGFs, in the actions of $1,25(OH)_2D_3$ in prostate cells.

In PC-3 and ALVA 31 cells, $1,25(OH)_2D_3$ decreases the availability of IGF by increasing the expression of its binding proteins IGFBP-3 and IGFBP-5 [161,162]. Boyle *et al*. [147] provided evidence that the up-regulation of IGFBP-3 expression by $1,25(OH)_2D_3$ is a necessary component of $1,25(OH)_2D_3$–mediated inhibition of LNCaP cell growth. $1,25(OH)_2D_3$ treatment increases IGFBP-3 mRNA levels. Importantly, addition of IGFBP-3 anti-sense oligonucleotides abrogates $1,25(OH)_2D_3$–mediated growth inhibition (Fig. 6). Immunoneutralization of the IGFBP-3 protein similarly
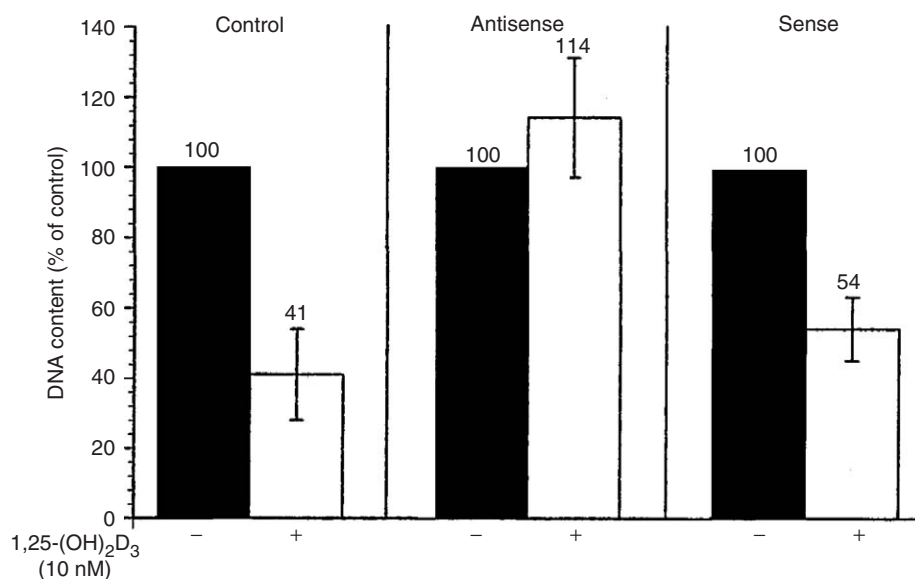
FIGURE 6   Abrogation of $1,25(OH)_2D_3$ mediated growth inhibition of LNCaP cells by IGFBP-3 anti-sense oligonucleotides. Cells were seeded in 96-well plates and grown in serum-free growth medium for 4 days with 10 nM $1,25(OH)_2D_3$ (+) or ethanol vehicle (−), along with 8 μg/ml of anti-sense or sense IGFBP-3 oligonucleotides. No oligonucleotides were added to the control group. DNA concentrations were determined at the end of the experiment and values in cells treated with vehicle for each group were defined as 100%. Values are shown as mean ± SE of three experiments. From Boyle *et al*. [147] with permission.

abrogates growth inhibition by $1,25(OH)_2D_3$. The data suggest that in LNCaP cells the growth inhibitory action of $1,25(OH)_2D_3$ depends on IGFBP-3 up-regulation. This study also demonstrates that the increase in the levels of the CDK inhibitor protein p21 elicited by $1,25(OH)_2D_3$ could be blocked by anti-IGFBP-3 antibodies showing that IGFBP-3 induction is necessary for the up-regulation of p21 expression by $1,25(OH)_2D_3$. IGFBP-3 expression in benign human prostatic epithelial cells has also been shown to be increased by $1,25(OH)_2D_3$. Nickerson and Huynh [163] demonstrated prostate regression in rats following the administration of the vitamin D analog EB 1089, which was associated with increases in the expression of several IGFBPs including IGFBP-3. The authors concluded that the prostate regression was related to alterations in the availability of IGFs as a result of increased production of IGFBPs. In recent studies Peng *et al.* characterized a VDRE in the promoter region on the IGFBP-3 gene [163a].

TGFβ and IGFBP-3 are pleiotropic factors that play an important role in the regulation of growth and differentiation in many cells [164–166]. They inhibit proliferation and induce apoptosis in prostate epithelial cells [164,166]. In PC-3 human PCa cells TGFβ has been shown to increase the expression of IGFBP-3 leading to growth arrest and apoptosis [165,166]. In NRP-152 cells, a nontumorigenic epithelial line derived from rat dorsolateral prostate, $1,25(OH)_2D_3$ was shown to induce the expression of TGFβ2 and TGFβ3. In these cells TGFβ appears to mediate growth inhibition and certain biological responses due to both retinoic acid and $1,25(OH)_2D_3$ [167]. The presence of VDRE sequences was demonstrated in the promoter of the human TGFβ2 gene [168].

E. Inhibition of Invasion and Metastasis

In addition to the inhibition of proliferation in malignant cells, $1,25(OH)_2D_3$ is also believed to play a role in tumor invasion and metastasis. $1,25(OH)_2D_3$ can inhibit the invasiveness of breast and lung carcinoma cells *in vitro* [169,170]. $1,25(OH)_2D_3$ decreases the tumor size and lung metastasis of the highly metastatic MAT LyLu and R3327-AT-2 Dunning PCa cells *in vivo* [115,126]. Schwartz *et al.* [171] showed that $1,25(OH)_2D_3$ and the analog 1,25-dihydroxy-16-ene-23-yne-cholecalciferol markedly inhibited the invasiveness of DU 145 human PCa cells through Amgel and also caused selective decreases in the secreted levels of matrix metalloproteinases (MMP)-2 and -9. A study by Sung and Feldman [172] showed that in DU 145 and PC-3 PCa cells, $1,25(OH)_2D_3$

inhibited invasiveness, cell adhesion, and migration to the basement membrane matrix protein laminin due in part to decreasing the expression of α6 and β4 integrins. In LNCaP and PC-3 cells, $1,25(OH)_2D_3$ and its analogs have also been shown to increase the expression of E-cadherin, a tumor suppressor gene whose expression is inversely correlated to the metastatic potential of the cells [131].

F. Angiogenesis

Angiogenesis or the process of new blood vessel formation is critical for tumor progression and metastasis. $1,25(OH)_2D_3$ has been shown to inhibit tumor cell-induced angiogenesis in mice [173,174]. Bernardi *et al.* [175] examined the ability of $1,25(OH)_2D_3$ to modulate angiogenic signaling in tumor-derived endothelial cells and observed a reduction in the angiogenic signaling molecule, angiopoietin-2. These studies support the hypothesis that angiogenesis inhibition plays a role in the antitumor effects of $1,25(OH)_2D_3$ [176].

G. Novel $1,25(OH)_2D_3$ Target Genes

$1,25(OH)_2D_3$, acting through the VDR, initiates its effects on cell growth and differentiation by the direct activation or repression of target genes. The identification of target genes is one of the goals of current research on vitamin D. Freedman and co-workers used a differential screening technique to isolate putative $1,25(OH)_2D_3$–inducible target genes during myeloid cell differentiation and identified genes encoding the CDK inhibitor p21 and the homeobox protein HoxA10 as vitamin D targets [143].

Feldman, Peehl, and colleagues recently performed cDNA microarray analyses in primary human prostatic epithelial cells derived from normal prostate or an adenocarcinoma of Gleason grade 3/3 as well as LNCaP cells to identify molecular targets of $1,25(OH)_2D_3$ involved in the regulation of prostate epithelial cell growth [70,113]. Table I lists some of the putative responsive genes in LNCaP cells as well as primary prostatic epithelial cells derived from normal prostate or PCa. Several interesting and noteworthy observations have emerged from these studies. 24-hydroxylase, the classical $1,25(OH)_2D_3$–inducible target gene, is maximally up-regulated in both normal and cancer-derived primary cell cultures. In contrast, in LNCaP cells the basal and induced levels of 24-hydroxylase are extremely low as has been shown earlier by Northern blot analysis [72]. As discussed earlier in Section III.B.1, lower levels of

24-hydroxylase in LNCaP cells makes them sensitive to the growth inhibitory effects of $1,25(OH)_2D_3$. In LNCaP cells, the expression of the IGFBP-3 gene shows the highest fold-increase after $1,25(OH)_2D_3$ treatment, which is in agreement with the previous study by these investigators showing that the up-regulation of IGFBP-3 expression is essential for $1,25(OH)_2D_3$–mediated growth inhibition in these cells [147]. No regulation of IGFBP-3 could be detected in primary cells consistent with the fact that primary cells do not express IGFBP-3. Several of the genes regulated by $1,25(OH)_2D_3$ in LNCaP cells, such as NDRG1 and 15-hydroxyprostaglandin dehydrogenase (Table I), have also been demonstrated to be androgen-responsive [177,178]. These findings are in accord with earlier data showing that in LNCaP cells the growth inhibitory action of $1,25(OH)_2D_3$ is androgen-dependent [80].

TABLE I Summary of Results from cDNA Microarray Analysis of $1,25(OH)_2D_3$ (10 nM) Treatment of LNCaP Cells, and Primary Cultures of Normal and Prostate Cancer Cells

Gene	Fold increase at 6 h	Fold increase at 24 h
LNCaP cells		
Insulin-like growth factor binding protein-3	2.4	33.2
N-myc downstream regulated (NDRG1)	1.1	3.3
Liprin β 2	1.9	2.7
Hydroxy prostaglandin dehydrogenase 15-(NAD)	0.95	2.7
Prostate differentiation factor	2.6	2.6
ATP-binding cassette, subfamily A, member 5	1.0	2.4
Claudin 4	2.0	2.2
Normal prostate epithelial cell strain		
Cytochrome P450, subfamily XXIV (vitamin D 24-hydroxylase)	79	83
Metallothionein 1H	12.7	5.3
Metallothionein 1L	12	2.8
Metallothionein 1G	9.2	6.7
Metallothionein 1E	6.2	2.4
Bone morphogenetic protein 6	3.9	3.0
Dual specificity phosphatase 10	3.1	2.0
Apoptosis inhibitor 4 (survivin)	3.1	2.9
Thioredoxin reductase 1	3.1	1.8
A kinase anchor protein (gravin) 12	2.8	1.6
Purinergic receptor P2Y, G-protein coupled 2	2.6	2.3
Carbonic anhydrase II	2.4	3.2
Hydroxy prostaglandin dehydrogenase 15-(NAD)	1.2	2.3
Prostate cancer epithelial cell strain		
Cytochrome P450, subfamily XXIV (vitamin D 24-hydroxylase)	78	46
Dual specificity phosphatase 10	3.4	0.51
Purinergic receptor P2Y, G-protein coupled 2	2.9	0.9
Bone morphogenetic protein 6	2.8	3.2
Thioredoxin reductase 1	2.8	1.6
A kinase anchor protein (gravin) 12	2.5	−1.4
Carbonic anhydrase II	2.1	4.1
Tumor necrosis factor (ligand) superfamily, member 11	1.0	4.3
MAD (mothers against decapentaplegic, drosophila) homolog 6	1.0	2.1
Superoxide dismutase 2, mitochondrial	1.0	2.0

These cDNA microarray analyses have revealed several novel putative vitamin D target genes in primary epithelial cells (Table I). In general, there is an appreciable overlap in the profiles of $1,25(OH)_2D_3$–regulated genes in normal and cancer-derived primary cells. In both of these cell types, the expression of dual specificity phosphatase 10 shows maximal up-regulation. Dual specificity phosphatase 10 inactivates mitogen activated protein kinase (MAPK), suggesting that an important feature of the growth inhibitory activity of $1,25(OH)_2D_3$ in these cells may be an inhibition of the growth-promoting effect of MAPK. Early up-regulation of the kinase anchoring protein gravin that is known to coordinate the localization of protein kinase C (PKC) and protein kinase A (PKA) is of interest and may be related to the recently reported effect of vitamin D on the packaging of PKC in chondrocytes [179]. These data are also supportive of the role of vitamin D as an antioxidant in primary prostate cells (see Chapter 45). Thioredoxin reductase 1, involved in redox balance, is an early response gene in both normal and cancer cells. Up-regulation of superoxide dismutatse 2 is also indicative of protection from oxidative damage. The regulation of the expression of metallothionein genes by $1,25(OH)_2D_3$ is different between the normal and cancer-derived primary cells, the former showing an up-regulation and the latter a significant down-regulation. Metallothioneins constitute the majority of intracellular protein thiols and as such are considered to act as cell survival factors. Up-regulation of metallothioneins in normal prostatic epithelial cells is consistent with the anti-apoptotic effect of $1,25(OH)_2D_3$ in these cells. It is also noteworthy that $1,25(OH)_2D_3$ up-regulates the expression of the anti-apoptotic protein survivin in these cells. Certain metallothioneins have been reported to be over-expressed in PCa [180] and hence a down-regulation of their expression in the cancer-derived cells may be therapeutically beneficial.

In summary, cDNA microarray analysis is a powerful tool that has revealed biologically important targets of $1,25(OH)_2D_3$ in prostate cells and has provided a starting point for additional investigations into the molecular mechanisms underlying the anti-cancer effect of $1,25(OH)_2D_3$ and its analogs.

VI. VITAMIN D IN COMBINATION WITH OTHER AGENTS

The efficacy of $1,25(OH)_2D_3$ in PCa therapy is directly related to the dose of $1,25(OH)_2D_3$ administered. Hypercalcemia becomes more frequent at higher concentrations of $1,25(OH)_2D_3$, which limits the maximum dose that can be given safely. Several vitamin D analogs (Section VIII of this book) exhibit increased potency as antiproliferative agents and have less hypercalcemic effects and therefore form a class of agents with a higher therapeutic index than the parent compound, $1,25(OH)_2D_3$. Another avenue to increase efficacy and decrease toxicity is to use a combination of agents that act by different mechanisms, at doses that are less than required when the agents are administered individually. This drug combination strategy has the advantage of limiting the toxicity associated with the individual drugs while obtaining additive and potentially synergistic therapeutic effects. Investigators have been studying the antiproliferative effect of vitamin D in prostate cancer in combination with other agents as discussed below.

A. Vitamin D and 24-hydroxylase Inhibitors

As detailed in Section III.B, a combination of $1,25(OH)_2D_3$ with inhibitors of 24-hydroxylase such as liarozole [97] (Fig. 3) or ketoconazole [101] increases the half-life of $1,25(OH)_2D_3$ in PCa cells and also leads to VDR up-regulation. The combination of $1,25(OH)_2D_3$ with 24-hydroxylase inhibitors therefore would allow the use of lower doses of $1,25(OH)_2D_3$ *in vivo* possibly reducing the hypercalcemic side effects. Ketoconazole, an imidazole derivative that inhibits mammalian P 450 enzymes, has been shown to exhibit growth inhibitory activity by itself in PCa cells [181], as well as in primary prostatic epithelial cells [101]. Ketoconazole has been shown to block testicular synthesis of androgens, and therefore has been frequently used to ablate androgen biosynthesis in PCa patients [182]. However, ketoconazole, in addition to blocking 24-hydroxylase [183], also blocks the activity of 1α-hydroxylase (Chapter 5) leading to low levels of $1,25(OH)_2D_3$, a major risk factor for osteoporosis and osteomalacia [69]. Indeed, administration of ketoconazole to normal men has been shown to result in a dose-dependent decrease in serum levels of $1,25(OH)_2D_3$ [184]. Men on this ketoconazole-based androgen ablation therapy would therefore be at a great risk for metabolic bone disease and other side effects of vitamin D deficiency. Adding $1,25(OH)_2D_3$ or analogs to therapeutic regimens that include ketoconazole would be beneficial as it would restore circulating levels of active vitamin D [101]. However, the clinical use of ketoconazole and $1,25(OH)_2D_3$ combination needs to be approached with caution. Ketoconazole inhibits multiple P 450 enzymes including those in the steroidogenic pathways leading to the synthesis of testosterone, cortisol, and aldosterone [182]. Adrenal insufficiency would exacerbate hypercalcemia. Therefore, patients

undergoing this combination therapy require careful monitoring for hypercalcemia and adrenal insufficiency in addition to the assessment of prostate cancer progression.

B. Vitamin D and Retinoids

The retinoids are comprised of vitamin A and its derivatives, including all-trans retinoic acid (ATRA), 9-cis retinoic acid (9-cis RA), and 13-cis retinoic acid (13-cis RA). These compounds are ligands for the retinoid receptors (RARs and RXRs) that act as nuclear transcription factors. RXRs are transcription factors that heterodimerize with RAR, VDR, and a variety of other nuclear transcription factors (see Chapters 11 and 13). In turn, these ligand-activated complexes regulate a variety of cellular processes including growth and differentiation.

Because of their role in controlling growth and differentiation, retinoids have been examined for potential anti-cancer activity in epithelial and nonepithelial malignancies, and may be particularly active in certain hematologic malignancies [185]. Prostate tissue expresses RARs and RXRs. Interestingly, cancer tissue appears to have lower concentrations of endogenous ATRA than normal prostate tissue, suggesting a possible rationale for a chemopreventive role for retinoids in prostate cancer [186]. Retinoids have also shown efficacy in controlling prostate cancer growth in animal models [187–189].

Several *in vitro* studies demonstrate synergistic activity between vitamin D and retinoids in prostate cells. ATRA enhanced the growth inhibitory effects of $1,25(OH)_2D_3$ on primary cultures of normal and malignant prostatic epithelial cells [190]. Although Esquenet *et al*. [81] did not find any cooperativity between ATRA or 9-cis RA and $1,25(OH)_2D_3$ on LNCaP cell growth in the presence of androgens, Blutt *et al*. [144] reported that $1,25(OH)_2D_3$ and 9-cis RA inhibited the growth of LNCaP cells in a synergistic manner and caused the accumulation of these cells in the G_1 phase of the cell cycle. Zhao *et al*. [110] also showed synergistic inhibitory activity between $1,25(OH)_2D_3$ and 9-cis RA on the growth of LNCaP cells. In LNCaP cells, $1,25(OH)_2D_3$ and 9-cis RA increased the expression of AR mRNA, and the effect of both the hormones on AR expression was additive [110]. Various other retinoids have been shown to sensitize prostate cancer cells to $1,25(OH)_2D_3$ or its analogs [191–193]. These studies provide a rationale for testing the use of combinations of vitamin D compounds with retinoic acid derivatives in PCa therapy.

C. Vitamin D and Glucocorticoids

Glucocorticoids have been widely used in the treatment of advanced PCa and have been part of PCa therapy in several randomized trials (for a review see [194]). Although glucocorticoids do not induce apoptosis in PCa cells, they exhibit growth inhibitory effects [195,196]. Glucocorticoids may exert anti-tumor effects *in vivo* on PCa by producing a negative feedback on the pituitary gland, leading to a decrease in both testicular and adrenal androgens [194]. They appear to inhibit prostate cell growth *in vitro* by modulating growth factors such as lipocortin, TGFβ-1, urokinase-type plasminogen activator, and interleukin-6 [194]. However, in PCa cells carrying promiscuous AR mutations (see Section I.B), some glucocorticoids can inappropriately act through the AR and stimulate cell growth [18,19,197].

Combination therapy with glucocorticoids and vitamin D compounds in general enhances the anti-tumor activity of vitamin D. However, Peehl *et al*. [190] made the observation that hydrocortisone at μM concentrations significantly reduced the antiproliferative effect of $1,25(OH)_2D_3$ in primary prostatic epithelial cells. In a murine squamous cell carcinoma model system, dexamethasone has been shown to reduce $1,25(OH)_2D_3$–mediated hypercalcemia and enhance the anti-tumor activity of $1,25(OH)_2D_3$ *in vitro* and *in vivo*, possibly due to the up-regulation of VDR levels [198]. Further investigations using this model system suggest the involvement of *Erk* and *Akt* signaling pathways in the antiproliferative effects of $1,25(OH)_2D_3$ and dexamethasone combinations [199]. Ahmed *et al*. [200] have shown that $1,25(OH)_2D_3$ significantly increases mitoxantrone and dexamethasone mediated growth inhibition in PC-3 PCa cells *in vitro*, and the combination of $1,25(OH)_2D_3$ plus mitoxantrone and dexamethasone causes significantly greater tumor regression in PC-3 xenograft-bearing mice. These studies have prompted Trump and colleagues to test the combination of $1,25(OH)_2D_3$ and dexamethasone in clinical trials (see also Chapter 97).

D. Vitamin D and Peroxisome Proliferator-activated Receptor (PPAR) Ligands

The PPARs, like VDR, RXR, and RARs, are members of the steroid receptor superfamily of proteins. Ligands for PPARγ exhibit anticancer activity against a wide variety of neoplastic cells including PCa cells, and *in vivo* studies in animal models also demonstrate the anti-cancer and chemopreventive capabilities of

these ligands [201]. PPARγ ligands have been shown to inhibit the growth of PC-3 human PCa cells [202,203], producing morphological changes consistent with a less malignant phenotype [202]. The PPARγ ligand troglitazone also inhibited the growth of PC-3 xenograft tumors in immunodeficient mice [202]. The PPARγ ligand rosiglitazone has been shown to inhibit the growth of primary prostatic epithelial cells and increase the expression of genes encoding differentiation-associated secretory proteins such as adipophilin in these cells [204]. Hisatake *et al.* [205] showed that PPARγ ligands inhibited the activation of the PSA gene by androgens in LNCaP cells and thereby suppressed PSA production by these cells. They also demonstrated that oral administration of troglitazone reduced the increase in velocity of PSA levels in a patient with occult recurrent PCa. Mueller *et al.* [206] conducted a phase II clinical study in patients with advanced PCa and found an unexpectedly high incidence of prolonged stabilization of PSA in patients treated orally with troglitazone. Although combinations of vitamin D compounds and PPARγ ligands have not been tested in cell culture, animal models, or clinical trials, the existing data provide a rationale for studying the effect of this combination in PCa therapy and chemoprevention of PCa.

E. Vitamin D and Soy Isoflavones

Soy is a rich source of isoflavones such as genistein and daidzein. An inverse correlation between consumption of soy in the diet and PCa incidence has been shown in epidemiological studies [207]. Soy-derived phytoestrogens have been demonstrated to inhibit the growth of PCa cells in culture [27,208]. Genistein decreases the expression of AR mRNA and protein and inhibits the transcriptional activation of the PSA gene in LNCaP cells [209]. Genistein also decreases AR expression in the dorsolateral prostate of rats [210]. *In vivo* studies in animal models have demonstrated an inhibitory effect of dietary genistein on the development of PCa [211,212] and a decrease in plasma testosterone levels in genistein-fed rats [213].

Several studies have shown interactions between phytoestrogens and the vitamin D axis in prostate cells. $1,25(OH)_2D_3$ and genistein have been shown to synergistically inhibit the growth of human primary prostatic epithelial cells and LNCaP cells [214]. The combination of these two agents causes arrest of the primary prostate cells in the G_0/G_1 phase as well as the G_2M phase of the cell cycle [214]. Farhan *et al.* [215] have shown that genistein and other isoflavanoids modulate the availability of $1,25(OH)_2D_3$ in DU 145

cells by inhibiting the activity of 24-hydroxylase and 1α-hydroxylase. The inhibition of 24-hydroxylase appears to be at the transcriptional level while that of 1α-hydroxylase involves deacetylation. A recent study by Weitzke and Welsh [216] reported an up-regulation of the transcription of the VDR promoter as measured by reporter gene activity and also demonstrated an increase in VDR protein expression in breast cancer cells following phytoestrogen treatment. Thus, a combination of vitamin D compounds and phytoestrogens such as genistein may be beneficial in PCa treatment as genistein can potentiate the growth inhibitory activity of vitamin D by the various mechanisms mentioned above.

F. Vitamin D and Chemotherapeutic Drugs

Several studies in cell cultures and animal models as well as clinical trials (see Section VII) have demonstrated the potential utility of vitamin D and its analogs as agents that can enhance the antiproliferative and cytotoxic effects of conventional chemotherapeutic drugs. Combined administration of $1,25(OH)_2D_3$ or its analog with platinum compounds such as carboplatin or cisplatin has been shown to result in a marked enhancement of growth inhibition in breast cancer [217] and prostate cancer cells [218] over that seen with the platinum compound alone. Wang *et al.* [219] showed that pretreatment of breast cancer cells with $1,25(OH)_2D_3$ or all-trans retinoic acid (ATRA) significantly lowers the threshold for cell killing by the chemotherapy drugs paclitaxel and adriamycin. The vitamin D analog EB1089, in combination with paclitaxel, effectively inhibits the growth of MC-7 breast cancer cell growth *in vivo* [220]. A recent study by Hershberger *et al.* [221] showed that treatment of murine squamous carcinoma cells and PC-3 PCa cells *in vitro* with $1,25(OH)_2D_3$ prior to paclitaxel caused significantly greater growth inhibition than either agent alone. In PC-3 tumor-bearing mice, $1,25(OH)_2D_3$ pretreatment similarly enhanced the tumor inhibitory effect of paclitaxel [221]. The molecular basis for the enhanced anti-tumor activity of this combination appears to be the increase in the expression of the cell cycle inhibitor p21 by $1,25(OH)_2D_3$, rendering the cells more susceptible to paclitaxel-induced apoptosis [221]. Paclitaxel cytotoxicity has been similarly shown to be increased in breast and colon cancer cells when p21 expression is specifically perturbed [222,223]. The ability of vitamin D to reduce cell survival signals may explain why it can enhance the anti-tumor activity of mechanistically diverse cytotoxic agents such as platinum compounds and taxol derivatives. These data

clearly support the combined use of vitamin D compounds and cytotoxic drugs in the treatment of solid tumors such as PCa.

A recent *in vitro* study demonstrates synergistic inhibition of the growth of PCa cells by vitamin D or its analogs in combination with histone deacetylase inhibitors such as sodium butyrate and trichostatin A [224]. Both *in vitro* and *in vivo* investigations thus far support the use of vitamin D or analogs in combination with other growth inhibitory agents in clinical trials of PCa prevention and/or treatment.

VII. CLINICAL TRIALS

Investigators have undertaken clinical trials in cancer patients to evaluate the safety and efficacy of treatment with vitamin D or its analogs (see Chapter 97 for details). Based on the extensive data developed from cell culture and animal models, there have been several strategies attempted. First, because it is an approved drug, $1,25(OH)_2D_3$ (calcitriol) has been used and administered in as high a dose as tolerated, limited by hypercalciuria or hypercalcemia. Second, trials are attempting to use less hypercalcemic analogs with a wider therapeutic window than calcitriol. Third, calcitriol has been administered intermittently in very high doses where it apparently can still cause its antiproliferative effects and only transient hypercalcemia that, thus far, does not appear to cause toxicity. Fourth, calcitriol or analogs are being used in combination therapy with agents that may enhance the antiproliferative activity while reducing its hypercalcemic tendency.

Osborn *et al.* [225] reported a small phase II trial of calcitriol in 13 patients with hormone-refractory metastatic PCa. No objective responses (>50% reduction in serum PSA levels or >30% reduction in measurable tumor mass) could be seen, and the median time to progression was 10.6 weeks. A pilot study by Gross *et al.* [226] used increasing concentrations of calcitriol to treat seven patients with early recurrent PCa following radiation or surgery. At the beginning of the trial, the patients showed no evidence of metastasis, and the only sign of recurrent disease was rising levels of serum PSA. Calcitriol doses were limited to 2 µg/day due to hypercalciuria in all patients. The doubling time of serum PSA before and after calcitriol treatment was compared in each patient. As shown in Table II, in all seven patients the rate of PSA rise was substantially decreased by calcitriol, and in the case of one patient, the serum PSA levels substantially decreased, registering a negative doubling time. Due to the formation of small asymptomatic renal stones, calcitriol therapy was discontinued in two of the seven patients. Withdrawal from therapy resulted in the resumption of the rise in PSA with the doubling time returning to the values seen before therapy was initiated (Table II). This study provides evidence that vitamin D could be effective in slowing the progression of PCa, based on PSA values. However, when therapy was discontinued, the resumption of growth at pretherapy levels suggests that calcitriol was only cytostatic and did not kill the cancer cells. These early trials underscore the fact that development of hypercalciuria or hypercalcemia would preclude the use of very high doses of calcitriol and therefore limit its therapeutic benefit that may only be realized at high doses.

TABLE II   PSA Doubling Time in Months Before and After $1,25(OH)_2D_3$ Treatment of Men with Early Recurrent PCa

Patient no.	Before therapy	During therapy	p Value	Therapy discontinued
1	17.1	−3.3	0.005	10.8
2	5.5	12.5	< 0.0001	2.1
3	12.0	37.6	0.009	13.7
4	10.8	15.8	0.002	
5	23.2	43.0	0.029	
6	12.5	27.4	0.02	
7	4.1	9.4	0.0009	

Patients completed 6 to 15 months of $1,25(OH)_2D_3$ therapy starting with a dose of 0.5 µg/day administered orally. The dose was increased on 0.25 µg increments weekly to a maximum dose of 2.5 µg/day, depending on individual calcemic and calciuric responses. Serum samples were collected for PSA measurements weekly during the dose escalation phase and then monthly. PSA doubling time was calculated before, during, and after therapy.

Adapted from Gross *et al.* [226] with permission.

Smith *et al*. [227] conducted a phase I trial in patients with advanced solid tumors to determine the maximum tolerated dose of calcitriol administered subcutaneously every other day (QOD) and found hypercalcemia occurring at the 10 μg QOD dose. A recent study by Beer *et al*. [228] tested the effect of calcitriol at a very high oral dose once weekly in patients with a rising PSA after prostatectomy or radiation therapy. This study found the weekly oral administration of 0.5 μg/kg calcitriol to patients on a low calcium diet to be safe and the median PSA doubling time increased from 7.8 months to 10.3 months. This dosage, approximately 70-fold the physiologic replacement dose of calcitriol, suggests that very large bolus intermittent therapy may be a means to enhance the anti-cancer actions of calcitriol while avoiding its side effects. The hypercalcemia returned to normal within 24–36 hours, and thus far renal stones or impairment of kidney function have not been noted.

As discussed in Section IV, several structural analogs of calcitriol that are more potent as antiproliferative agents but exhibit less hypercalcemic effects are being developed and characterized. A phase I study of the analog 1α-hydroxyvitamin D_2 (paracalcitol) administered orally to patients with advanced hormone refractory PCa recommended a dose of 12.5 μg/day given continuously for use in phase II trials [229].

Investigators have also been studying the efficacy of calcitriol in treatment of men with PCa in combination with other agents as discussed in detail in Section VI. Johnson *et al*. [230] have initiated phase I and phase II trials of calcitriol, either alone or in combination with other agents. Preclinical data indicate that glucocorticoids potentiate the anti-tumor effects of calcitriol and alleviate some of the hypercalcemic side effects (see Section VI.C). One of the trials initiated by Johnson *et al*. [230] is a phase II study of calcitriol in combination with dexamethasone in 43 patients with AIPC. Altogether, 80% of the patients have exhibited a slowing in the rate of increase in serum PSA, and 34% have had stable disease or decrease in PSA (>50% reduction) levels. Patients with bone pain at study entry have had pain relief.

As discussed in Section VI.F, cell culture and animal studies indicate that calcitriol and its analogs can enhance the cytotoxic effects of conventional chemotherapeutic drugs, and investigators have begun testing these combinations in phase I and phase II trials. Trump, Johnson, and co-workers are conducting two phase I trials testing 1,25(OH)$_2$D$_3$ in combination with carboplatin or paclitaxel. In the study testing calcitriol and carboplatin, no dose-limiting toxicity has been seen up to calcitriol doses of 13 μg/day orally for three days every four weeks with carboplatin, and the study

continues [230]. In the second trial, paclitaxel is given with escalating doses of calcitriol every day for three days per week for a period of six weeks, and so far no dose limiting toxicity has been encountered [231].

Beer *et al*. [232] are conducting a phase II study to evaluate weekly oral administration of very high dose calcitriol (0.5 μg/kg) on day 1 followed by intravenous administration of docetaxel on day 2 in patients with AIPC, repeated for six consecutive weeks of each eight-week cycle. In a recent report Beer *et al*. [233] showed that the combination of a once weekly oral high-dose calcitriol and weekly docetaxel was a well-tolerated regimen for patients with AIPC. Thus far 22 out of 37 patients exhibit a > 75% reduction in serum PSA at the end of an eight-week treatment cycle, confirmed four weeks later. The results of this study indicate that PSA and measurable disease response rates, as well as time to progression and survival, are more promising with the combination approach when compared to phase II studies of the single agent docetaxel in AIPC. To confirm these encouraging findings these investigators have started a placebo-controlled, double-blinded randomized comparison of docetaxel plus calcitriol to docetaxel alone.

The result of the various clinical investigations so far support the potential promise of exploiting vitamin D compounds alone or in combination with other agents to control PCa progression and further studies are clearly warranted.

VIII. SUMMARY AND CONCLUSIONS

A number of studies have established the role of 1,25(OH)$_2$D$_3$ as an antiproliferative agent in normal and malignant prostate cells. Investigations using animal models have also demonstrated the anti-tumor effects of 1,25(OH)$_2$D$_3$ and its less hypercalcemic analogs. The mechanisms underlying the anti-cancer effects of vitamin D compounds in PCa cells are varied and cell-specific and include growth arrest, apoptosis, pro-differentiation effects, and modification of growth factor activity. Some studies suggest additional effects to inhibit invasiveness and anti-angiogenesis, but there are less data on these issues. 1,25(OH)$_2$D$_3$ interacts with androgen signaling in PCa cells possibly enhancing its prodifferentiating activity. Investigators are making progress in identifying 1,25(OH)$_2$D$_3$-regulated genes and understanding their role in the mediation of the above-mentioned effects. Such studies would unveil novel 1,25(OH)$_2$D$_3$–responsive genes and provide new therapeutic targets.

Administration of pharmacological doses of calcitriol to men with PCa results in hypercalcemia and hypercalciuria, which limits the concentration of

calcitriol that can safely be administered to patients. Consequently, structural analogs of calcitriol that effectively activate the VDR but exhibit less hypercalcemic effects are being developed and evaluated as potential anti-cancer agents. A challenging area of research involves defining the mechanisms by which various vitamin D analogs maintain potent growth inhibitory effects and yet are less able to induce hypercalcemia. Intermittent dosing regimens with very high boluses of calcitriol are an interesting approach that apparently inhibits PCa while not causing persistent hypercalcemia. Combinations of calcitriol or its analogs with inhibitors of 24-hydroxylase or other growth-inhibitory molecules such as retinoids, glucocorticoids, or cytotoxic chemotherapeutic drugs permit the use of vitamin D and the other agents at relatively lower doses, thereby avoiding toxic side effects while achieving synergistic antitumor effects. Several investigators are carrying out clinical trials to determine whether calcitriol or analogs singly or in combination with other agents can prevent the progression of PCa. Investigators are also examining the role of vitamin D compounds as chemopreventive agents.

We believe that progress in these areas of research will result in rational drug design and lead to the development of more potent and safer vitamin D analogs. It remains to be determined what will be the most effective use of calcitriol and its analogs in terms of dosage regimen, combination with other agents, and phase of the disease in which it will be most useful in the treatment and/or prevention of PCa.

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Vitamin D and Colon Cancer

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I. INTRODUCTION

A. Colonic Tumor Prevention by Vitamin D

1. EPIDEMIOLOGICAL EVIDENCE

As early as 1980 Garland *et al.* [1] proposed that vitamin D may be a protective factor against colorectal cancer. This hypothesis was based on the observation that the geographic distribution of colon cancer mortality in the U.S.A. was highest in regions where the population was least exposed to solar radiation. UV-B is responsible for vitamin D production in the skin, and serum levels of 25-hydroxyvitamin D_3 (25-(OH)-D_3) are a direct reflection of sunlight exposure, of the use of sun blockers, and of skin pigmentation [2]. Thus, low serum 25-(OH)-D_3 levels generally found in African-Americans probably are a reflection of reduced vitamin D synthesis due to high melanin concentrations in the skin [3]. This population segment also has enhanced incidence of colorectal, breast, and prostate cancer [4]. The link between colorectal cancer incidence and solar radiation was later confirmed by Freedman *et al.* [5] and by several large studies comparing southern and northern parts of the U.S.A. [6]. Recently, Grant [7] suggested that actually 20–30% of colorectal cancer incidence is due to insufficient exposure to sunlight (see Chapters 90 and 91).

A follow-up study by Garland *et al.* [8] in almost 2000 males demonstrated that risk of colorectal cancer correlated inversely with dietary vitamin D and calcium intake. Further studies on dietary vitamin D and calcium (see [9]) appeared to confirm these data. When Garland *et al.* [10] demonstrated in blood samples from a Washington County population that a serum 25-(OH)-D_3 concentration of 20 ng/ml or more decreased threefold the risk of colon cancer, a direct connection between serum vitamin D levels and colon cancer incidence appeared to be established. However, in this study, cancer incidence was evaluated only 1–8 years after blood sampling. In a 19-years prospective study, it was subsequently established that a dietary intake of more

than 3.75 micrograms vitamin D/day was associated with a 50% reduction in the incidence of colorectal cancer [11]. The same nested case-control study based on a cohort of more than 25,000 individuals demonstrated that a moderately elevated serum concentration of 25-(OH)-D_3 (65–100 nmol/l) was associated with a highly significant reduction in colorectal cancer incidence [11].

Several later studies, however, provided ambiguous results: a large 10–17 year retrospective study of Washington County residents did not provide any link between colorectal cancer incidence and serum levels of 25-(OH)-D_3, or of 1,25-dihydroxyvitamin D_3 (1,25-(OH)$_2$-D_3) evaluated prior to disease occurrence [12]. When serum 25-(OH)-D_3 concentrations in patients with colonic neoplasia were compared with those of noncancer patients, no correlation with the disease was found either [13]. Tangrea *et al.* [14], however, did find that the estimated relative risk of large bowel cancer decreased with increasing serum 25-(OH)-D_3 levels, and that the association was most pronounced for rectal cancer in a nested case-control study within a Finnish clinical trial cohort. It is highly interesting that in this large study levels of 1,25-(OH)$_2$-D_3 again did not correlate at all with colon cancer risk.

One of the very few studies demonstrating a correlation between low circulating levels of 1,25-(OH)$_2$-D_3 (below 26 pg/ml, a level considered to be below normal) and enhanced risk was found in the prospective Nurses' Health Study. A higher risk of distal colorectal adenomas was found in individuals with lower than 26 pg 1,25-(OH)$_2$-D_3/ml serum [15]. In another study by Niv *et al.* [16], which was marred by the small number of patients involved, a steady reduction of serum 1,25-(OH)$_2$-D_3 was observed in parallel with advancing tumor stages, but not with the biological tumor grade.

A plethora of studies has been based on semiquantitative food frequency questionnaires, which often involved not only vitamin D but dairy food intake as well. Therefore, calcium ingestion may be a confounding factor in these evaluations. The consensus is that,

if vitamin D consumption has any preventive effect on colorectal cancer incidence, it is a very modest one. However, this protective effect might be augmented by intake of multivitamin supplements (see [17–23]). In this respect, the argument was raised that solar radiation or nutritional vitamin D intake, for instance by consuming fatty fish, would not be sufficient to effectively prevent the occurrence of colorectal tumors. Indeed, in a later study Garland *et al.* [24] suggested that intake of at least 800 IU (20 micrograms) of vitamin D_3 together with 1000 mg calcium would be needed to significantly reduce the incidence and mortality rates of human colon cancer.

2. CONCLUSION

Thus, cumulative epidemiological evidence suggests that there is a direct correlation between reduced colorectal cancer incidence and sunlight exposure, low skin pigmentation, nutritional vitamin D intake, and high serum levels of 25-(OH)-D_3. This association could be strengthened by vitamin D supplementation. In a recent human pilot study, Holt *et al.* [25] demonstrated for the first time that rectal crypt proliferation was inversely correlated with 25-(OH)-D_3 levels in serum. This indicates, though in an indirect way, that low 25-(OH)-D_3 levels may be indeed associated with colorectal cancer incidence. However, no relationship between serum 1,25-(OH)$_2$-D_3 and disease occurrence was convincingly apparent in any of the cited studies, except at very low serum 25-OH-D concentrations.

II. MOLECULAR BASIS OF VITAMIN D ACTION ON NEOPLASTIC COLONOCYTES

Since the 1980s, 1,25-(OH)$_2$-D_3 has been recognized as a potent cellular antiproliferative and prodifferentiating agent in the colon. More recently, there has been intense interest in its effects on apoptosis, malignant cell invasion, and metastasis. The classical signaling pathway is via a nuclear vitamin D receptor (VDR), which is a transcription factor (see [26]). The existence of a separate "membrane" receptor has also been suggested [27]; however, recent data from a VDR knockout mouse provide good evidence that this purported receptor is of minor importance in the intestine [28].

A. The Vitamin D Receptor in Normal and Malignant Colon Cells

In 1982, Frampton *et al.* [29] provided evidence that the VDR was present in many human cancer cell lines and also in all colonic cell lines they investigated. Since then, the presence of the VDR has been extensively studied in a variety of colon cancer cell lines.

Giuliano *et al.* [30] showed in Caco-2 cells that the VDR was functional in these colon cancer cells and that expression was increased when Caco-2 cells became confluent and differentiated in culture. Interestingly, Zhao and Feldman [31] demonstrated convincingly that, at least in HT-29 cells which were differentiated by chemical means, VDR abundance was actually decreased with decreased proliferation and increased differentiation. Brehier and Thomasset [32] found no specific binding of 1,25-(OH)$_2$-D_3 in differentiated HT-29 cells. Harper *et al.* [33] also found smaller amounts of the receptor in galactose-grown, i.e. differentiated, HT-29 cells when compared with undifferentiated (glucose-grown) HT-29 cells. In Caco-2 cells, however, there was strong expression of the VDR upon differentiation. Conversely, activation of proliferation in these cells by epidermal growth factor (EGF) resulted in down-regulation of the expression of the high affinity receptor [34].

The question of whether the VDR was more highly expressed in proliferating or differentiated colon cancer cells was further studied by Shabahang *et al.* [35]. Their conclusion was that the more differentiated the colonic cell lines were, the higher was their VDR expression. They also evaluated presence of the VDR in human malignant colonic tissue. In the majority of these tumors, they found lower expression of VDR than in tumor-adjacent normal mucosa from the same patient; however, the number of cases analyzed was small (12 patients). Lointier *et al.* [36] investigated VDR expression in 23 human tumor tissue specimens and in adjacent normal-appearing mucosa. They did not observe any difference in VDR distribution between normal right and left colon, or the rectum. However, they did find the receptor in most of the normal tissue specimen, but only in very few of the adenocarcinomas. Notably, all positive adenocarcinomas were of the well-differentiated (low grade) type. Vandewalle *et al.* [37] demonstrated significantly higher VDR expression in transformed colon than in normal tissue in the proximal and distal colon, but not in the rectum. These data were accrued by binding studies with tissue homogenates. Cross *et al.* [38] were the first to demonstrate by immunoblotting that VDR protein expression was increasing during the transition from normal mucosa to polyps and during progression into malignancy. In rather advanced tumor stages, however, expression was diminished or disappeared completely. This suggests that colon cancer cells express the VDR as long as they retain a certain level of differentiation. This could explain why their data differed from those obtained by

TABLE I Semi-quantitative Evaluation of VDR and EGFR mRNA Expression in Epithelial Cells

| | Normal adjacent mucosa | Adenocarcinoma | |
		Low grade	High grade
VDR	17.5 ± 1.4	$125.0 \pm 19.7^*$	46.3 ± 5.5
EGFR	58.2 ± 5.5	$122.0 \pm 18.1^*$	$142.5 \pm 27^*$

In situ hybridization (IHS) reactivity scores were calculated by multiplying the percentage of receptor positive cells by the average signal intensity. Data are mean $\pm$ SEM. $*$ indicates statistically significant difference from respective IHS score in normal adjacent mucosa at $p < 0.01$ (using Student's unpaired t test).

some other groups, who did not take into account tumor staging and grading. Further studies by Sheinin *et al.* [39], on a larger number of human colon tissues demonstrated convincingly, by *in situ* techniques, that in normal colon VDR expression is weak and positivity is found mainly in luminal cells, i.e. differentiated crypt cells. During tumor progression, the number of VDR-positive colonocytes increased dramatically in parallel with epidermal growth factor receptor (EGFR) expression, and it reached its maximum in low-grade (well to moderately differentiated) tumor tissue, whereas in high grade cancers (G3/4, low differentiation or undifferentiated tissue), VDR expression was very low. In contrast, EGFR expression rose even further in undifferentiated tumors (Table I).

When establishing primary cultures from human premalignant and malignant colonic tissue at diverse stages of tumor progression, Tong *et al.* [40] have shown a mosaic pattern of VDR expression in colon cells. This demonstrated that a large fraction of cells isolated from human colon tumors, but not all, expressed the VDR and thus could respond to the genomic action of vitamin D compounds. Cross *et al.* [41] and Kállay *et al.* [42] demonstrated by immunoblotting and RT-PCR in colon tissue derived from 61 patients that there was indeed little VDR present in normal tissue, and that expression rose during colon tumor progression.

In Fig. 1, VDR expression is shown in parallel with a proliferation marker, Ki-67. Barely any VDR expression is found in a (G3/4) tumor with cells at a low differentiation level (Fig. 1C), when it is was compared with expression in normal colon mucosa (Fig. 1A) or in a moderately-differentiated (G2) tumor (Fig. 1B). Low VDR expression in normal mucosa (Fig. 1A) is paralleled by low Ki-67 positivity mainly in the lower crypt area (Fig. 1D). With enhanced VDR expression in low grade cancer (Fig. 1B) there is also markedly increased Ki-67 positivity (Fig. 1E), whereas in high grade tumors with strong proliferation, there is almost no more VDR positivity apparent (Fig. 1C and F).

These, and data from other laboratories, led to the suggestion that vitamin D receptor expression could be used as a predictive marker of biological behavior in human colorectal cancer [43].

The importance of the VDR for prevention of colonic hyperproliferation and of potential tumorigenesis was demonstrated by Kállay *et al.* [44] in the VDR knockout mouse model established by the group of Kato [45]. Complete loss of the VDR resulted in colonic hyperproliferation, cyclin D1 elevation, and
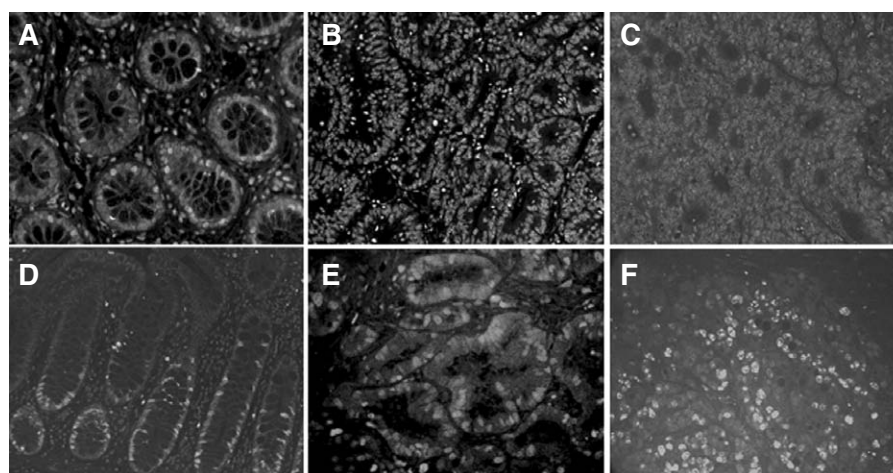
FIGURE 1   Evaluation of vitamin D receptor (**A**, **B**, **C**) and Ki-67 (**D**, **E**, **F**) expression in normal and malignant human colon. **A**, **D**: Normal colon mucosa, **B**, **E**: Moderately differentiated (G2) adenocarcinoma, and **C**, **F**: Adenocarcinoma of the colon at low differentiation (G3/4). (Cross and co-workers, unpublished data).

a dramatic increase of DNA damage mainly in the distal colon (as measured by 8-hydroxy-2′-deoxyguanosine accumulation). This suggests that, at least in this animal model, growth control by 1,25-(OH)$_2$-D$_3$ is highly effective in the distal [44], but not in the proximal colon, and may be essential for maintenance of normal growth conditions of mucosal cells (see also Fig. 2).

Recently, data from a population-based case-control study of colon cancer suggested that normal molecular variants of the VDR gene, i.e. polymorphisms of the VDR, might be related to the development of colon cancer [46]. The different variants could have less or more transcriptional activity upon binding of the hormonal ligand (see Chapter 68). Epidemiological studies to

evaluate the association of these variants with diet and lifestyle factors are needed.

B. Effect of 1,25-(OH)$_2$-D$_3$ and of Vitamin D Analogs on Proliferation, Differentiation, and Apoptosis of Colonic Cells

The vitamin D receptor is known to exert its antimitotic and prodifferentiating effect by binding vitamin D metabolites and analogs. This not only maintains normal growth of the colonic crypt, but potentially can prevent the progression into premalignancy (see [42]). Interestingly, it has been observed recently [47] that there may be other paths of colon cancer prevention following VDR activation: the VDR can also bind the enteric carcinogen lithocholic acid, and can activate its detoxification via transcriptional induction of cytochrome P450 enzymes (see Chapter 53).

The following sections will present, with some selectivity, experimental evidence for the effects of various vitamin D compounds on proliferation, differentiation, and apoptosis of colon cells in in vitro systems, in animal models, and in human studies.

1. In Vitro Models

a. Action of 1,25-(OH)$_2$-D$_3$ In 1987 Lointier et al. [48] demonstrated that 10 nM 1,25-(OH)$_2$-D$_3$ inhibited growth of the LoVo colon cancer cell line under serum-free conditions. Brehier et al. [32], by evaluating induction of brush border hydrolase activity, demonstrated the differentiating effect of 1,25-(OH)$_2$-D$_3$ in HT-29 cells. Harper et al. [33] observed a decrease in the growth rate of HT-29 cells at the low concentration of 10 pM 1,25-(OH)$_2$-D$_3$. Cross et al. [49–51] provided extensive evidence that 1–10 nM 1,25-(OH)$_2$-D$_3$ decreased growth and increased activity of the differentiation marker alkaline phosphatase in the human colon adenocarcinoma-derived cell line Caco-2. Induction of hyperproliferation of Caco-2 cells resulted in 100-fold higher sensitivity to the antimitotic prodifferentiating action of the secosteroid hormone [49]. Evidence for a dose-dependent reduction of proliferation and increased alkaline phosphatase activity in Caco-2 cells was provided also by Halline et al. [52]. Responsiveness of primary cultures obtained from human normal colon, colon adenomas, and carcinomas to 1,25-(OH)$_2$-D$_3$ was demonstrated for the first time by Tong et al. in 1998 [40]. The proliferative rate of adenoma cells was initially twice that of cells obtained from normal mucosa. When adenoma cells were treated with 10 nM of the secosteroid, their mitotic rate was reduced to that of normal colonocytes in culture. Carcinoma-derived primary cultures responded to 1,25-(OH)$_2$-D$_3$ and vitamin D analogs in
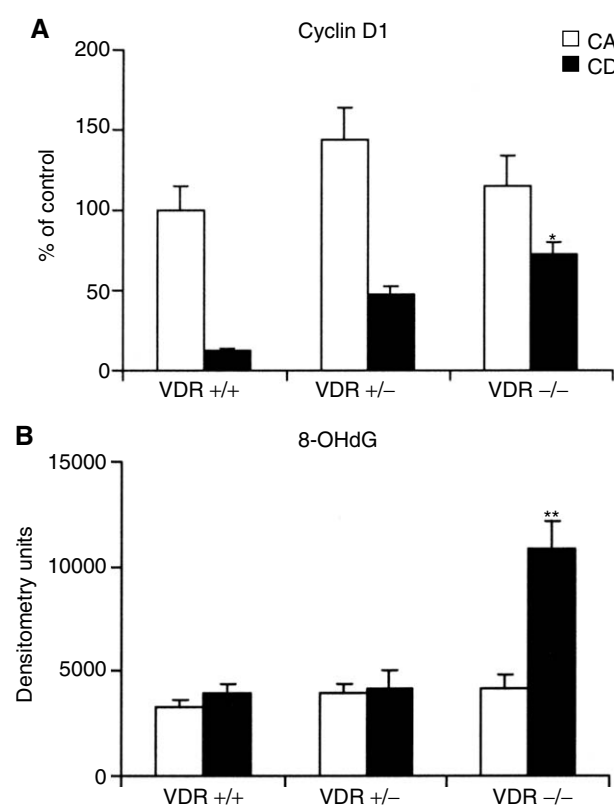

FIGURE 2 **A**: Expression of Cyclin D1 protein by immunoblotting in the ascending (CA) and descending (CD) colon of wild-type (VDR+/+), heterozygous (VDR+/−), and VDR knockout (VDR−/−) mice. Values are expressed as mean ± SD, n = 5 animals per genotype. Statistically significant differences compared to the VDR+/+ group are indicated as * (p<0.05) (Student's t test). **B**: Immunohistochemical evaluation of 8-OHdG expression in ascending (CA) and descending (CD) colon of wild-type (VDR+/+), heterozygous (VDR +/−), and VDR knockout (VDR−/−), mice. For quantification three 35 mm photo slides were taken randomly from each sample and were analyzed by NIH Image freeware. Values are expressed as mean ± SD, n = 5 animals of each genotype. Statistical significance was indicated as **(p<0.01). (Student's t test). 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

a concentration-dependent manner with respect to proliferation and differentiation.

Since colorectal tumors are frequently under mitotic control by EGF or TGF-α, and during human colon tumor progression EGFR expression is up-regulated in parallel to that of the VDR [39] (see also Table I), Tong *et al.* [53] evaluated the interaction of EGF with the 1,25-(OH)$_2$-D$_3$. Their data demonstrated a mutual modulation of the VDR by the hormones, which resulted in enhanced activity of 1,25-(OH)$_2$-D$_3$ in EGF-treated Caco-2 cells. Bareis *et al.* [54] pointed out that only well-differentiated colonic cell lines or primary cultures were also able to respond to 10 nM 1,25-(OH)$_2$-D$_3$ by growth reduction. Their data showed that EGF treatment of a differentiated Caco-2 clone, but not of an undifferentiated one, increased VDR expression (see also Fig. 3). This suggested that VDR mediated growth inhibition by 1,25-(OH)$_2$-D$_3$ would be effective only in differentiated human colorectal carcinomas.

Franceschi *et al.* [55] found that 1,25-(OH)$_2$-D$_3$ was able to stimulate fibronectin synthesis in colon cancer

A

B

FIGURE 3   **A**: Evaluation of [^{3}H]-thymidine incorporation into DNA in Caco-2/AQ and Caco-2/15 clones. Cells were treated for 48 hours with 10 nM 1,25-(OH)$_2$-D$_3$. Data are accumulated from two separate experiments and are presented in percent of ethanol control (mean ± SD; n = 6 per group; **: $p < 0.01$). **B**: Western blot analysis of VDR expression in Caco-2/AQ and Caco-2/15 cells with and without exposure to 10 ng EGF for 48 hours.

cell lines, indicating that the secosteroid may also play a restrictive role in tumor metastasis. Recently, Palmer *et al.* [56] demonstrated that only VDR–positive clones derived from the human colon carcinoma cell line SW480 were able to respond to the differentiating action of 1,25-(OH)$_2$-D$_3$. Conversely those clones that lacked the VDR did not respond. This vitamin D–induced differentiation process resulted in induction of E-cadherin and of other adhesion proteins, and promoted the translocation of β-catenin from the nucleus to the plasma membrane. 1,25-(OH)$_2$-D$_3$ also repressed β-catenin/TCF-4 transcriptional activity and modulated target genes in a manner opposite to that of β-catenin. Wilson *et al.* [57] demonstrated in HT-29 cells the significance of this effect with respect to regulation of the *c-myc* oncogene: elevated β-catenin/TCF signaling due to mutations in the adenomatous polyposis coli (APC) gene resulted in increased transcriptional activity of *c-myc*, one of the early immediate genes initiating cell cycle traverse. 1,25-(OH)$_2$-D$_3$ induced transcriptional blockage that resulted in decreased c-myc expression.

TGF-β, a well recognized growth inhibitor of normal colonocytes, is no longer active in human colon cancer cells. However, 1,25-(OH)$_2$-D$_3$ treatment activated TGF-β signaling in Caco-2 cells, and enhanced abundance of the type 1 TGF-β receptor [58]. Thus, the secosteroid sensitizes Caco-2 cells to the growth-inhibitory action of TGF-β1.

According to very recent data by Thompson *et al.* [59], transcriptional activity of the liganded VDR may result also in induction of cytochrome P450 detoxification enzymes, which may contribute to colonic chemoprotective mechanisms by detoxification of enteric carcinogens. Though several reports have been published on rapid and membrane based signal transduction mechanisms following exposure of colon cancer cells to 1,25-(OH)$_2$-D$_3$ (see [60–62]), none is of specific relevance yet for colon cancer prevention or therapy by 1,25-(OH)$_2$-D$_3$.

b. Vitamin D Analogs   It is becoming increasingly evident that adjuvant treatment of colorectal cancer patients with 1,25-(OH)$_2$-D$_3$ for its antimitotic prodifferentiating activity would necessitate a pharmacological dose, which would have the classical adverse consequences, namely hypercalcemia, soft tissue calcification, and nephrocalcinosis. Therefore, over the past decades, more than 400 analogs of vitamin D have been chemically synthesized and their biological properties have been systematically explored, calcemic effects have been quantified, and cell-differentiating and antimitotic potential have been evaluated (for reviews see [63,64] and Chapters 80–88). Action of some of these analogs on colon cancer cells will be reviewed here.
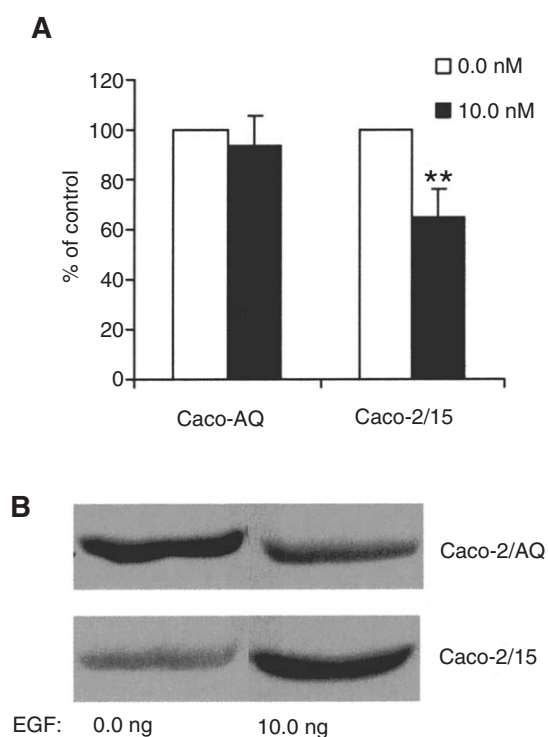
Cross *et al.* [51] evaluated concentration-dependent growth inhibition in relation to hypercalcemic potential of two side chain-modified synthetic vitamin D analogs (Ro23-4319, Ro23-7553). Ro23-7553, a 16ene-23yne side chain-modified vitamin D analog, was tenfold more effective than the 1,25-$(OH)_2$-D_3 in suppressing growth of Caco-2 cells; however, it was also tenfold more potent in stimulating calcium release from cultured mouse calvariae. With respect to intestinal calcium absorption the analogs were rather less effective than the parent hormone. Several other studies [65–69] evaluated only differential growth inhibition of colon cancer cells by a variety of analogs, and the main conclusion was that side chain-modified analogs improve activity of the secosteroid maximally by a factor 10, while their hypercalcemic activity is still moderately high. Only the 1β-(hydroxymethyl) congeners of the natural hormone, though still possessing significant antimitotic activity in spite of their low affinity for the VDR [70], did not promote osteoclast differentiation *in vitro*. This suggests that at nanomolar doses they would not cause hypercalcemia in human studies [71].

Oh *et al.* [72] implied that the antiproliferative activity of EB1089 in HT-29 cells was, at least in part, due to decreased secretion of IGF-II and increased concentrations of the IGF-II binding protein IGFBP-6. Tanaka *et al.* [73,74] observed enhanced differentiation of HT-29 cells upon treatment with a combination of sodium butyrate and vitamin D analogs and proposed this combination as a differentiation-based therapy for the clinical management of human colon cancer. Evaluation of Ro25-6760 action on HT-29 cells demonstrated significant growth inhibition, apoptosis induction, with enhanced expression of p21[Waf1] and G1/Go cell cycle arrest [75]. An increase of the proapoptotic protein BAK induced by the vitamin D analog EB1089 in colon cell lines also suggested a mechanism of action involving apoptosis [76]. Another vitamin D analog was shown to increase expression of the cdk inhibitors p21[Waf1] and of p27[Kip1] independent of changes in pRB [77].

2. ANIMAL MODELS FOR COLORECTAL TUMOR PREVENTION BY VITAMIN D COMPOUNDS

As early as 1988, Pence and Buddingh [78] evaluated the effect of 2000 IU vitamin D_3/kg diet on 1,2-dimethyl-hydrazine-induced colon carcinogenesis in male rats. Development of cancer was promoted with 20% corn oil in the diet. Their data suggested that only in animals on a high fat diet (i.e. the promoter) did vitamin D_3 significantly reduce tumor incidence. Subsequent work by Kawaura *et al.* [79], who instilled intrarectally lithocholic acid in rats with N-methyl-N-nitrosourea-induced colonic tumors, demonstrated that 1α-(OH)-D_3 as well

was inhibiting promotion of tumorigenesis. Apparently, without exogenous promoters, vitamin D compounds did not affect colonic carcinogenesis and did not interfere with formation of bile acid profiles [80]. Comer *et al.* [81] also provided evidence that dietary levels between 250–10,000 IU vitamin D_3/kg diet did not alter carcinogen-induced tumor incidence without prior promoter treatment, while work by Belleli *et al.* [82] using 400 ng 1,25-$(OH)_2$-D_3 per rat (an exceedingly high dose) suggested a protective effect of the secosteroid if it was delivered before the mutagen. When the protective action of vitamin D in combination with dietary calcium on carcinogen-induced colon tumors in rodents was investigated [83,84], it became apparent that both substances affected cellular kinetic indices, i.e., tumor size and not tumor incidence, and that their mode of action appeared to be a cooperative one.

Newmark and Lipkin [85] introduced the concept of a nutritional stress diet for mice designed to represent the human Western diet, which is deficient in calcium and vitamin D, and rich in phosphate and fat. This diet led to hyperproliferation and hyperplasia in the rodent colon and, when fed long enough, to functional and structural modifications in the colon mucosa similar to those found in humans at increased risk for colonic neoplasia [86]. Mokady *et al.* [87] demonstrated that rats fed the Western stress diet had an enhanced response to tumor induction by a carcinogen, whereas supplementary vitamin D_3 abrogated this tumor induction. (See also Chapter 91.)

Several studies addressed the question of the efficacy of vitamin D analogs in rodent colon tumor models [88–91]. In these it was claimed that blood calcium levels generally were not raised by the analogs and that the development of aberrant crypt foci, a putative neoplastic lesion, or of carcinogen-induced tumors was significantly reduced by vitamin D compounds. The most pronounced inhibition was found if analogs were administered after carcinogen treatment. The well-known vitamin D analog EB1089 was used in a human colon cancer LoVo cell xenograft study in a nude mouse model and led to 50% inhibition of tumor growth [92]. Another xenograft study with human colorectal cancer cells differing in VDR content demonstrated that tumor growth of VDR-positive cells was reduced in a concentration-dependent manner by 1,25-$(OH)_2$-D_3 and the hexafluorinated analog Ro25-6760, whereas growth of VDR-negative xenografts was not [93]. Tanaka *et al.* [94] used a novel analog, DD-003, to inhibit HT-29 human colon cancer cell growth under the renal capsule of immunodeficient mice. Interestingly, PKC isoform expression was significantly altered in precancerous lesions in rat colon after treatment with Ro24-5531 [95].

While some of these vitamin D–derived compounds appear to be quite effective in reducing tumor size and tumor burden, hypercalcemia is still sometimes detected. The general consensus is clear: vitamin D analogs do inhibit colon carcinogenesis specifically when administered in the postinitiation phase by reducing colonocyte proliferation. A possible additional mechanism of action for these substances in rodent models might be inhibition of angiogenesis, as was demonstrated by Iseki *et al.* [96], and by inhibition of metastasis [97]. Recently, Wali *et al.* [98] demonstrated in rat colon that azoxymethane-induced aberrant crypt foci and tumors expressed enhanced levels of cyclin D1, of cyclooxygenase-2, and of inducible nitric oxide synthase, as well as reduced E-cadherin levels. These changes were significantly inhibited by a fluorinated vitamin D analog.

Studies on tumor prevention by $1,25\text{-}(OH)_2\text{-}D_3$ and its analogs were recently extended to the Apc(min) mouse by Huerta *et al.* [99]. They observed a significant decrease in total tumor burden while serum calcium in the group treated with analogs was only moderately elevated. These results suggest that such analogs may ultimately have utility as in humans chemopreventive agents at least in population groups at high risk for colon cancer, if serum calcium concentration is constantly monitored.

C. Clinical Studies

In 1992, Thomas *et al.* [100,101] evaluated *in vitro* crypt cell production rate (CCPR) in rectal tissue obtained from familial adenomatous polyposis (FAP) patients. 1 μM–100 pM $1,25\text{-}(OH)_2\text{-}D_3$, as well as the synthetic vitamin D analogs MC-903 and EB-1089, reduced CCPR in explant cultures by approximately 50%, whereas EGF increased CCPR by 100% [100]. This indicated for the first time that human colorectal tissue would indeed respond to $1,25\text{-}(OH)_2\text{-}D_3$ and its analogs in a similar manner to cell and animal experiments.

Accumulating experimental evidence from *in vitro* and from animal studies suggested that the analog EB-1089 was potentially useful for human treatment due to weaker calcemic side-effects while still maintaining high antimitotic, prodifferentiating, and apoptotic activity. In this respect, a promising phase I study with EB-1089 in patients with advanced colon and breast cancer was initiated [102]. An initial evaluation of this study [103] showed that hypercalcemia was still seen in patients receiving 17 microgram/day EB-1089 for 10 to 234 days. Hypercalcemia was reversible by discontinuing administration of the substance or by reducing the amount, and a tolerable dose for most patients was established at 7 microgram/day. There were no complete or partial responses, but 6 out of 21 patients on treatment for more than 90 days showed stabilization of their disease. The only other human trial was performed on FAP patients who had previously undergone colectomy but had upper gastrointestinal polyps. In this double-blind randomized crossover trial, the effectiveness of sulindac, a specific inhibitor of cyclooxygenase-2, was compared with that of calcium in combination with calciferol. While sulindac treatment resulted in reduction of the crypt cell proliferation index in gastric epithelium but not in duodenal mucosa, calcium and calciferol had no effect whatsoever [104].

D. Conclusion

Experimental results show quite clearly that, while $1,25\text{-}(OH)_2\text{-}D_3$ and also some of its analogs indeed have antimitotic and prodifferentiating activity in colon cancer cells *in vitro*, their use *in vivo*, especially in the human patient, has not yet been well explored. While administration at low doses does not appear to be very effective, the high nanomolar concentrations needed to inhibit growth frequently are prohibitively hypercalcemic.

Epidemiological data have not reliably supported the hypothesis that in humans serum $1,25\text{-}(OH)_2\text{-}D_3$ at the highest physiological range showed a negative correlation with colorectal tumor incidence (see Section I.A.1). However, there is apparently a negative correlation between high levels of $25\text{-}(OH)\text{-}D_3$, the proliferative index of crypt cells [25], and colorectal cancer incidence. While this obviously favors the population group living at latitudes with high incident sunshine, also those consuming vitamin supplements could have higher levels of the precursor of the active metabolite. This precursor could conceivably be used by colon cells for extrarenal synthesis of $1,25\text{-}(OH)_2\text{-}D_3$, which may function as an autocrine/paracrine cell cycle regulator in the colon. Evidence for this new concept of organ-localized accumulation of $1,25\text{-}(OH)_2\text{-}D_3$, which would not influence its serum levels, will be discussed in the following sections.

III. VITAMIN D METABOLISM IN NORMAL AND NEOPLASTIC COLON CELLS

A. Human Colon Cancer Cell Lines and Primary Cultures

In 1990, Tomon *et al.* [105] were the first to demonstrate vitamin D catabolism in an *in vitro* intestinal cell

model, the human colon adenocarcinoma-derived cell line Caco-2. These cells did not exhibit constitutive 25-(OH)-D$_3$-24-hydroxylase (CYP24) activity. Catabolic activity was inducible upon treatment with 100 nM 1,25-(OH)$_2$-D$_3$. While, at this early date, only conjectures about extrarenal synthesis and degradation of 1,25-(OH)$_2$-D$_3$ existed, its role in growth control in the intestine was already well established. Early data by Birge *et al.* [106] demonstrated, that in rats dosed with vitamin D, mucosal cell turnover was accelerated and there was an approximately 20% increase in villus height, whereas in vitamin D–deficient rats, villus height was blunted. Thus, there is a trophic as well as a differentiating influence on intestinal morphology, which, at the time, was attributed to serum 1,25-(OH)$_2$-D$_3$ that had been synthesized in the kidney from the precursor 25-(OH)-D$_3$. However, in retrospect this growth regulation could also have been attributed to extrarenal intestinal synthesis of 1,25-(OH)$_2$-D$_3$.

It was only much later in 1997 when Cross *et al.* [107] demonstrated in Caco-2 cells the conversion of the precursor 25 OH D$_3$ into 1,25-(OH)$_2$-D$_3$. They found constitutive expression of the 25-D$_3$-1α-hydroxylase (CYP27B1) in almost any growth phase of this cell line, and the sequential metabolism/catabolism of the secosteroid along the C-24 and C-23 oxidative pathways. It is therefore conceivable that human colon cells can control their growth via 1,25-(OH)$_2$-D$_3$ in an autocrine/paracrine manner dependent upon presence of the vitamin D receptor. Bischof *et al.* [108] provided evidence that distinct oxidation pathways for 1,25-(OH)$_2$-D$_3$ catabolism were used by two Caco-2 clones differing in their level of differentiation.

Colonic metabolism of vitamin D was subsequently evaluated in a variety of primary cultures, which were established from human colon tumors at different grades and stages. Such cultures are more closely related to human physiology and more appropriate to verify evidence obtained from cell lines. Figure 4 compares 25-(OH)-D$_3$ metabolism in two different human colonic cancer cell types. From high performance liquid chromatography (HPLC) analysis, it was obvious that COGA-13 cells, which were isolated from a G2/3 human adenocarcinoma of the right colon, had no innate 1α-hydroxylase activity, which, however, was pronounced in Caco-2 cells (Fig. 4A and 4B). Caco-2 cells responded with reduced 1α-hydroxylation and enhanced 24-hydroxylation to 1,25-(OH)$_2$-D$_3$ treatment (Fig. 4 C),
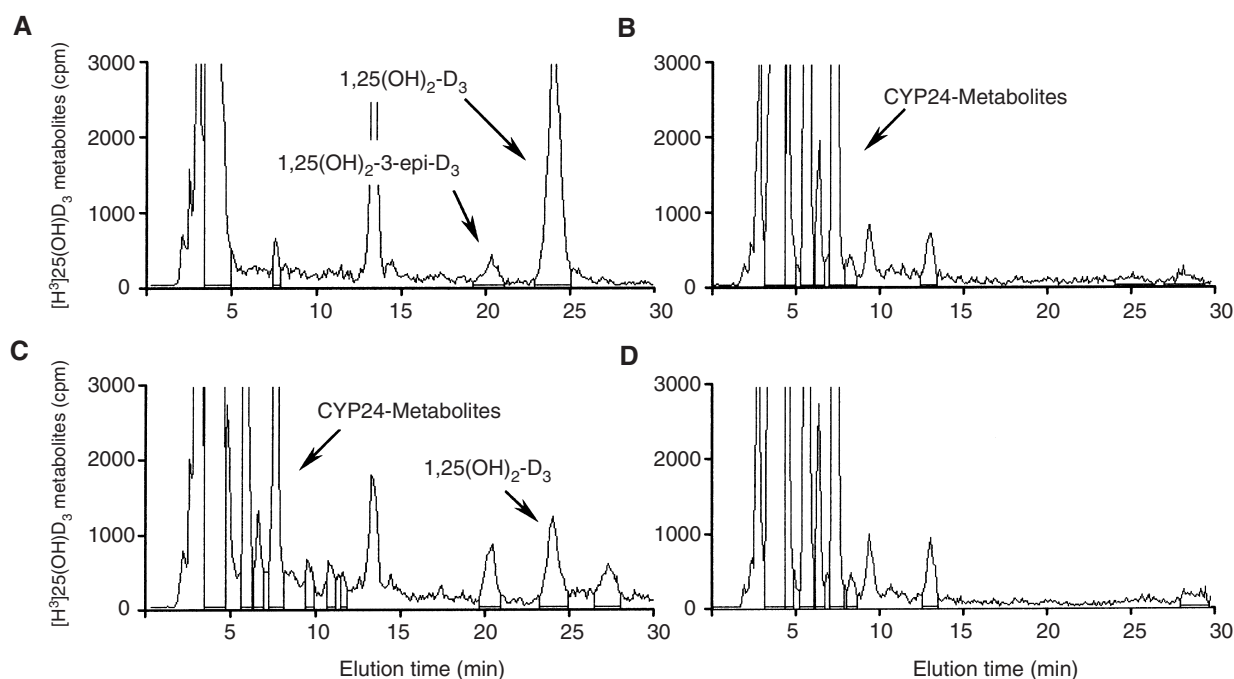

FIGURE 4   Comparison of HPLC profiles of 25-(OH)-D$_3$ metabolism in human colonic cancer cells. Cells were incubated with the precursor 25-(OH)-[26,27-methyl-^{3}H]-D$_3$. The 1α-hydroxylated compounds appear after 20–25 minutes elution time, whereas the 24-hydroxylated metabolites are seen much earlier between 5–10 minutes elution time, just after the precursor peak. **A**: Untreated Caco-2 cells have mainly 1α-hydroxylated metabolite production and almost no 24-hydroxylase activity. **B**: Untreated COGA-13 cells have constitutively high 24-hydroxylase activity and no 1α-hydroxylation. **C**: In Caco-2 cells treatment with 10 nM 1,25-(OH)$_2$-D$_3$ results in significant down-regulation of 1α-hydroxylase activity and significant increase of 24-hydroxylation. **D**: In COGA-13 cells, the same treatment yields no detectable effects on 25-(OH)-D$_3$ metabolism (Cross and co-workers, unpublished data).

whereas COGA-13 cells were insensitive, probably because their maximal 24-hydroxylation capacity had already been reached (Fig. 4D) (see also Section III.A.1).

These results support the hypothesis that it could be the degree of differentiation of cells that determines their ability for $1,25\text{-}(OH)_2\text{-}D_3$ synthesis or catabolism (see also [54]). Even more direct evidence for extrarenal vitamin D metabolism present in cancer patient-derived colon tissue was also provided. Cells were isolated from freshly excised human colon tumors, as well as from the adjacent normal mucosa outside the tumor border. After testing cells for general metabolic activity, they were immediately processed for HPLC assays. Bareis *et al.* [109] demonstrated unequivocally with this method that in human colon cancer cells there is very active vitamin D metabolism including both 24-hydroxylase and 1α-hydroxylase activity, whereas in the adjacent normal mucosa there is very little of either.

Bareis and co-workers also demonstrated by RT-PCR analysis in human colon tumor-derived primary cultures low CYP27B1 mRNA and high CYP24 mRNA expression. The latter, however, was not only present as the wild-type transcript, but also as a splice variant. The relationship between splice variants and enzymatic activity is as yet unknown [109].

There is accumulating evidence that human colon cancer cells express varying levels of the metabolic and catabolic vitamin D hydroxylases, supporting the hypothesis that the human colon tissue has differential capacity to accumulate the active hormonal metabolite. In addition, there are apparently unique regulatory processes that depend upon difficult to define levels of differentiation (see also Chapter 79).

1. REGULATION OF VITAMIN D METABOLISM AND CATABOLISM BY $1,25\text{-}(OH)_2\text{-}D_3$ AND EPIDERMAL GROWTH FACTOR *IN VITRO*

If cells in the human colon synthesize and catabolize $1,25\text{-}(OH)_2\text{-}D_3$ to different extents, this may be due to regulatory factors present in the cellular environment. The active hormone $1,25\text{-}(OH)_2\text{-}D_3$ could itself be present due to local synthesis, and this could result in regulation similar to the well known pathway in the kidney, i.e. down-regulation of 1α-hydroxylase activity and up-regulation of 24-hydroxylase activity. Consequently, treatment of Caco-2 cells for 48 hours with 10 nM $1,25\text{-}(OH)_2\text{-}D_3$ resulted in induced 24-hydroxylation and in considerable reduction of the 1α-hydroxylated peak, as measured by HPLC analysis (Fig. 4C). COGA-13 cells isolated from a high-grade colon adenocarcinoma, while expressing CYP24 strongly in the control, did not show any modulation

of their 24-hydroxylation pattern after treatment with the active vitamin D metabolite (Fig. 4D). Thus, there is, on the one hand, constitutive expression of this catabolic enzyme in cells derived from a colon tumor at a low differentiation level. On the other hand, in Caco-2 cells even under vitamin D treatment, there is still 1α-hydroxylation though reduced. However, there is also 24-hydroxylating activity. This clearly demonstrates the potential problem of hormone degradation, which might exist in colon tissue following localized synthesis of $1,25\text{-}(OH)_2\text{-}D_3$.

Two different clones derived from the Caco-2 cell line were analyzed (Caco-2/AQ with high proliferation, lower differentiation; Caco-2/15 with low proliferation, high differentiation). When their CYP27B1 expression levels after treatment with 10 nM $1,25\text{-}(OH)_2\text{-}D_3$ were compared, it became obvious that in Caco-2/AQ cells not only activity but also protein (and mRNA) level of CYP27B1 was down-regulated, whereas in Caco-2/15 cells it was increased [54]. Such up-regulated expression of CYP27B1 appeared to be typical for well-differentiated cell lines, and again suggests the primary importance of the biological grade of cells, which would determine synthesis and degradation of $1,25\text{-}(OH)_2\text{-}D_3$ in the human cancerous colon mucosa under physiological conditions.

Regulation of vitamin D metabolic and catabolic enzymes was also seen with EGF [54]. This latter observation may be of some physiological importance for colonic synthesis of vitamin D, since expression of the EGFR is well recognized to increase during colon tumor progression (see Table I and [39]) and transforming growth factor α (TGF-α), also a ligand for the EGFR, is an autocrine growth factor during tumorigenesis. Moreover, as described above in Section II.A.1.a, there is mutual regulation between the VDR and the EGFR in colon cancer cell lines (see also [110]).

B. Expression of CYP27B1 and CYP24 in Human Intestine

There is increasing evidence that during tumor progression differential cellular regulation of vitamin D metabolism and catabolism occurs possibly similar to the regulation demonstrated by Cross *et al.* [41] in colon cancer patients with respect to vitamin D receptor expression: VDR is elevated at the mRNA level very early during progression, i.e. in differentiated tumors, while late during progression VDR expression is significantly reduced [38,41]. Such a regulatory pattern points towards a physiological defense mechanism against tumor progression, which may fail during late stages.

1. EVALUATION OF mRNA EXPRESSION FOR CYP27B1 AND CYP24

Evaluation of tissue specimens from 50 colorectal adenocarcinomas by RT-PCR demonstrated convincingly that CYP27B1 mRNA was elevated in G1 and G2 tumors when compared with adjacent normal mucosa from the same patient, and also in comparison with colon mucosa from noncancer patients. In G3 tumors expression dropped to low levels [41]. This seemed to be true only for early stages of the disease (pT1-pT3), during late stage (pT4) disease both G2 and G3 tumors had low CYP27B1 mRNA expression [109]. Tangpricha *et al.* [111] studied CYP27B1 mRNA expression by real-time PCR in normal colon and colon tumors, though a quantitative evaluation was not possible due to the small number of patients. Recently, Ogunkolade *et al.* [112] showed CYP27B1 expression in colonic tissue also by real-time RT-PCR in a larger number of individuals. They did not confirm the increase of CYP27B1 in tumors compared with healthy colon samples from noncancer individuals described by Cross *et al.* [41] and Bareis *et al.* [109]. It has to be pointed out, however, that Ogunkolade *et al.* did not examine colonic tumors with respect to the biological grade of cells as was done in other studies [41,109].

Cross *et al.* [113] provided very recently the evaluation of CYP27B1 mRNA by real-time PCR in colon tumors and adjacent mucosa from 18 cancer patients, as well as in colon mucosa from 5 noncancer patients. All tumor patients had high to medium differentiated (G1, G2) primary adenocarcinomas. The authors clearly demonstrated that the normal mucosa from tumor as well as from nontumor patients had similarly low levels of CYP27B1 mRNA, whereas this was consistently increased in tumor tissue.

The discrepancies in results between different laboratories [41,109,113], and [111,112], are most likely caused by the fact, that (1) G3 tumors frequently have very low expression of CYP27B1 mRNA, and (2) that sometimes the adjacent "normal" mucosa of a G3 tumor displays high expression of CYP27B1 mRNA similar to that found in early colon tumors [42].

Bareis *et al.* [109] detected a transcript of CYP24 in human cancerous colon lesions as well as in the adjacent mucosa of the same patient. While CYP24 was consistently higher in tumor tissue than in adjacent normal tissue, they also found at least two transcripts of differing size, where the larger one contained an additional sequence with homology to intron 1. Expression levels of the smaller transcript appeared to be highest in late-stage high-grade tumors, whereas the larger one was present in low-grade highly-differentiated tumor tissue.

2. CYP27B1 PROTEIN EXPRESSION IN THE HUMAN COLON

Zehnder *et al.* [114] were the first to demonstrate by immunohistochemistry and immunoblotting extrarenal expression of the CYP27B1. Among the tissues investigated was also the human colon where staining was found in epithelial cells and apparently also in parasympathetic ganglia. They suggested that the discrete pattern of staining found in various organs in the human body emphasized a possible role for the hydroxylase as an intracrine modulator of vitamin D function in peripheral tissues.

When Cross and co-workers evaluated CYP27B1 protein expression in colon tissue samples (normal mucosa from noncancer patients, adenomas, low- and high-grade tumors and adjacent mucosa from the same patient) from 38 patients by immunofluorescence, they found that at least 50% of tumor patients were positive. This positivity depended completely upon histology of the tissue: As long as there were glandular differentiated structures present, these were positive for CYP27B1, even if the rest of the tissue consisted of cells of low differentiation and was negative for CYP27B1. Fig. 5A shows normal human colon mucosa with barely any positivity. Fig. 5B exemplifies strongly enhanced expression of CYP27B1 in a colon adenoma, while Fig. 5C shows strong expression in the small intestine (ileum).

a. Coexpression of CYP27B1, VDR, and Ki-67 Proteins in Human Colon Further evaluation of human colon tissue sections from cancer patients by immunofluorescence showed that many cells positive for the VDR were also positive for CYP27B1 as recently shown by Bises *et al.* [115]. However, there were also many cells only positive for the VDR, especially in normal and premalignant tissues. This again points to an autocrine/paracrine mode of action for the secosteroid.

While the VDR is obviously present also in normal mucosa where it may regulate mucosal function and growth with $1,25\text{-}(OH)_2\text{-}D_3$ as ligand, it is only during onset of malignant progression that CYP27B1 expression is strongly induced in more than 50% of patients. While the VDR frequently is found also in proliferating cells (there is coexpression with the Ki-67 antigen, see Fig. 1), CYP27B1 positivity is rarely found in Ki-67-positive cells (Cross *et al.*, unpublished observations).

While CYP27B1 positivity is barely apparent by immunofluorescence in the normal colonic mucosa, it is interesting to observe that in small intestine (ileum) much more positivity is present (Fig. 5C). This observation suggests that in the small intestine there is an innate defense against hyperproliferation due to high
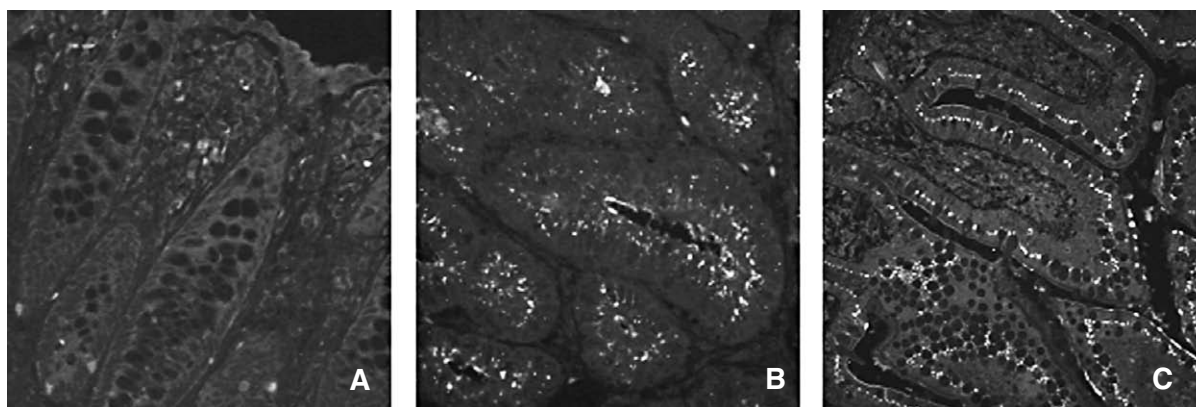
FIGURE 5   Immunofluorescence analysis of CYP27B1 protein in normal human colon mucosa **(A)**, colon adenoma **(B)**, and small intestine **(C)**. (Cross and co-workers, unpublished data).

local synthesis of the $1,25\text{-}(OH)_2\text{-}D_3$. This could, at least partially, be responsible for the low tumor incidence occurring in the human small intestine.

C. CYP27B1, CYP24, and VDR Expression in a Mouse Model

Kállay *et al.* [44] demonstrated in a mouse model that VDR expression was higher in the proximal than in the distal colon. Their results from a VDR-knockout (VDR-KO) mouse showed that it was mainly the distal colon that was negatively affected by the lack of genomic action of $1,25\text{-}(OH)_2\text{-}D_3$ [44]. However, CYP27B1 mRNA expression in proximal and distal mouse colon seemed quite similar, whereas CYP24 expression was much higher in the proximal than in the distal colon [116]. This implies that in the proximal colon less active vitamin D metabolite would be potentially available.

Recently Kállay *et al.* [117] demonstrated for the first time by RT-PCR that the regulation of CYP27B1 and CYP24 mRNA in the mouse colon is completely different from that in the kidney. In the VDR-KO mouse kidney, CYP27B1 mRNA expression is highly induced due to lack of the VDR and consequent ineffectiveness of $1,25\text{-}(OH)_2\text{-}D_3$ action, while, in the colon CYP27B1 expression is significantly down-regulated in parallel with enhanced proliferation (as shown by increased PCNA expression) (Fig. 6A, B).

These very recent results demonstrate again the unique aspects of extrarenal $1,25\text{-}(OH)_2\text{-}D_3$ synthesis, the completely different regulation in the colon in contrast to the one in the kidney, and also the potential importance this could have for colorectal cancer prevention.

IV. NUTRITIONAL REGULATION OF CYP27B1 AND CYP24

This new concept of extrarenal synthesis and catabolism of vitamin D in colonocytes should gain increasing importance for tumor prevention as well as for therapy, since $1,25\text{-}(OH)_2\text{-}D_3$ could act locally and prevent hyperproliferation and dedifferentiation without causing generalized hypercalcemia. However, some cells could respond to local accumulation of $1,25\text{-}(OH)_2\text{-}D_3$ with increased activity of CYP24 and decreased activity
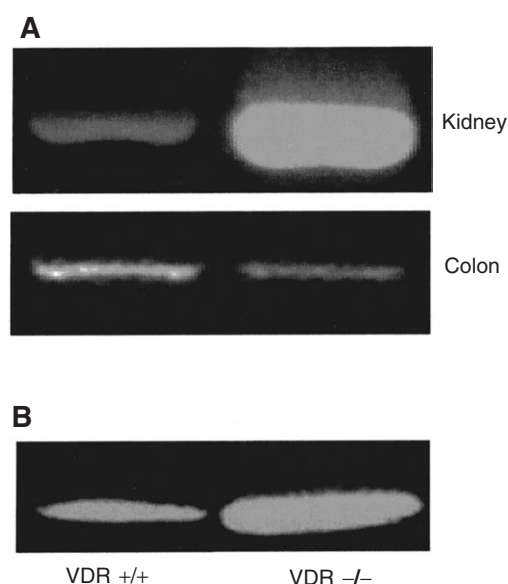

FIGURE 6   **A**: Comparison of the CYP27B1 status in the kidney and colon of wild-type (VDR+/+) and knockout (VDR−/−) mice. **B**: Expression of the proliferation marker PCNA in the colon of wild-type (VDR+/+) and knockout (VDR−/−) mice.

of CYP27B1. Therefore, the regulation of these colonic enzymes by, for instance, nutritional means to improve localized accumulation of $1,25\text{-}(OH)_2\text{-}D_3$ might be of some importance for cancer prevention and also for therapy of low-grade, early-stage tumors.

Considerable physiological evidence is accumulating for a protective effect of estrogenic substances against colorectal cancer incidence. At all ages, women are less likely than men to develop colon cancer, and post-menopausal hormone replacement therapy even further reduces colon cancer risk by up to 25%. Potter *et al.* [118] demonstrated lower risk of adenomatous polyps of the large bowel with hormone replacement therapy. In addition, in several colon cancer animal models, male rodents were shown to have higher tumor burden and increased aberrant crypt formation rates (see [119]), the latter being a typical precursor lesion of colorectal cancer. Very recently a large comprehensive study by the Women's Health Initiative Investigators [120] on physiological effects of hormone replacement therapy (HRT) was stopped, since most parameters that were assumed to be beneficially affected by HRT, were either negatively (for instance, breast cancer) or not at all affected. The only highly significant exceptions were reduction in incidence of colorectal cancer and of osteoporosis. Thus, the reluctance to use HRT for a longer period and for minor problems seems to be well founded. However, the observed reduction of colorectal cancer incidence needs to be explored further. The mechanism of action could conceivably involve modulation of the vitamin D system.

In the clinical situation, for instance, plasma $1,25\text{-}(OH)_2\text{-}D_3$ levels are elevated during human pregnancy and remain high postpartum in lactating women [120, 121] even beyond that component explained by increased DBP (see Chapter 51). In animal studies, female rats treated with estradiol benzoate daily for eight days had increased $1,25\text{-}(OH)_2\text{-}D_3$ concentrations in plasma, gut mucosa, and kidneys [123]. Interactions between vitamin D and estrogen have also been observed in murine colon carcinoma [124]. However, evidence that estrogenic substances indeed regulate vitamin D metabolism was still missing.

A. Regulation of $1,25\text{-}(OH)_2\text{-}D_3$ Synthesis by Phytoestrogens

Dramatically reduced incidence, especially of hormone-related cancers such as mammary and prostate tumors, has been linked to the consumption of a typical Asian diet, which contains high amounts of soy products and thus is rich in phytoestrogens. It is conceivable that these substances, through their potential to act as selective estrogen receptor modulators, could have an effect on vitamin D–related inhibition of tumor growth in the mammary and prostate gland. Interestingly, isoflavones belonging to the group of phytoestrogens have been shown to down-regulate estrogen receptor (ER) expression, which could lead to reduced estrogenic responses, i.e. protection against deleterious sex hormone effects in hormone-responsive tissues [125].

Foley and co-workers [126] suggested that malignant transformation of the human colon is associated with a marked diminution of ER-β expression, which is widely regarded to be the predominant ER-subtype in normal colonic tissue [127]. Phytoestrogens bind with high affinity to ER-β. This could indicate a possible protective mode of action for phytoestrogens in the colon, even though this requires further experimental evaluation.

Kállay *et al.* [128] demonstrated in a mouse model that soy feeding elevated CYP27B1 mRNA expression both in the proximal and distal sections of the colon. In contrast, CYP24 mRNA expression was considerably reduced by soy consumption. When they administered genistein, a well-known isoflavone, by gavage to mice, a similar effect was observed (Table II). This suggests that genistein, a major constituent of soy, is one of the nutritional substances that could be used to modulate vitamin D metabolism and catabolism.

These results imply potentially enhanced colonic synthesis of $1,25\text{-}(OH)_2\text{-}D_3$ in populations on high nutritional soy consumption, and conceivably enhanced protection against colorectal cancer. While, in breast and prostate tissue, phytoestrogens contained in soy may very well interfere with the action of estrogen itself, they could also stimulate extrarenal vitamin D synthesis

TABLE II Expression of CYP27B1 and CYP24 mRNA in the Murine Colon

	CYP27B1		CYP24	
	Right colon	Left colon	Right colon	Left colon
AIN 76A diet	242 ± 176	148 ± 76	111 ± 22	33 ± 9.4
Phytoestrogen diet	$468 \pm 135^*$	$460 \pm 182^*$	$23 \pm 11^*$	27 ± 6.7

Multiplex RT-PCR, i.e. simultaneous amplification of transcripts specific either for CYP27B1 or for CYP24, and a transcript specific for the epithelial cell marker cytokeratin 8, was carried out for semi-quantitative evaluation of respective mRNA expression levels. PCR products were analyzed on agarose gels. The level of CYP27B1 and CYP24 expression was correlated with that of the epithelial cell marker CK 8. Values are expressed as mean $\pm$ SD, n = 8 animals in each group. Statistical significance is indicated as * ($p < 0.05$).

in such tissues, enabling the steroid hormone to exert its well recognized antimitotic, prodifferentiating action [129].

V. CONCLUSION

While epidemiology has provided strong support for the concept, that vitamin D could be a prevention factor during colon tumorigenesis, it was only the serum level of the precursor 25-(OH)-D$_3$ and not 1,25-(OH)$_2$-D$_3$ itself that correlated convincingly with human colorectal tumor incidence. While 1,25-(OH)$_2$-D$_3$ is known to prevent proliferation and to induce differentiation and apoptosis in colonocytes, pharmacological doses are necessary, regardless whether it is tested in an *in vitro* or an *in vivo* system. Such high concentrations, however, are prohibitively hypercalcemic. While some analogs may maintain their effectiveness as cell cycle regulators at 10–100-fold lower doses than the parent compound, even these concentrations could result in hypercalcemia in patients. It therefore appears unlikely that any of these compounds will ever be used for preventive purposes. However, in late stage colon cancers, they may very well prove to stabilize the disease.

Recent data have demonstrated extrarenal synthesis of the secosteroid in the colon. Physiological regulation of vitamin D metabolic and catabolic hydroxylases in normal and malignant human colonic tissue suggests a role for the locally accumulated hormone in prevention of tumor progression. In addition, during low-grade early-stage malignancy, colonic synthesis of 1,25-(OH)$_2$-D$_3$ could potentially provide a block to progression, if 1,25-(OH)$_2$-D$_3$ catabolism could be inhibited.

Renal or colonic 1,25-(OH)$_2$-D$_3$ synthesis and catabolism is differentially regulated. While lack of vitamin D action due to absence of the VDR results in elevated expression of CYP27B1 in the kidney, the same enzyme is down-regulated in the colon, probably due to enhanced proliferation of the tissue. This implies that there may exist substances that could enhance extrarenal 1,25-(OH)$_2$-D$_3$ accumulation without affecting renal synthesis. Recent results allude to such action mechanisms for phytoestrogens. Genistein, a phytoestrogen and major component of soy, can induce expression of CYP27B1 and reduce that of CYP24 in the mouse colon. The involvement of phytoestrogens in the regulation of the vitamin D system could conceivably explain the observation that women have less colorectal cancer incidence than men, probably due to their higher estrogenic background. Consumption of phytoestrogens, however, could also be acceptable for men to protect against colorectal cancer incidence.

Acknowledgment

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Vitamin D and Hematological Malignancy

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I. OVERVIEW OF HEMATOPOIESIS

Hematopoiesis is the process that leads to the formation of the highly specialized circulating blood cells from bone marrow pluripotent progenitor stem cells. These stem cells are the most primitive blood cells, and they have the ability to either self-replicate or differentiate. They are regulated by a feedback system and are affected by various stimuli such as bone marrow depletion, hemorrhage, infection, and stress. They produce more mature "committed" cells that are able to proliferate and differentiate into cells of different lineages, acquiring specific functional properties (Fig. 1).

The pluripotent stem cell common to granulocytes, erythrocytes, monocytes, and megakaryocytes is called the *colony-forming unit-GEMM (CFU-GEMM)*, and the committed cells giving rise to the lineage specific cells are assayed *in vitro* as erythroid burst-forming units (BFU-E), megakaryocyte colony-forming units (CFU-MK), and granulocyte-monocyte colony-forming units (CFU-GM). Each of these stem cells has cell surface receptors for specific cytokines. Binding of cytokines to these receptors stimulates secondary intracellular signals that deliver a message to the nucleus to stimulate proliferation, differentiation, and/or activation.

The CFU-GMs, in the presence of cytokines, undergo a differentiation program progressing to granulocytes and monocytes. The growth factors acting primarily on the granulocyte-macrophage pathway are granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF). The GM-CSF also stimulates eosinophils, enhances megakaryocytic colony formation, and increases erythroid colony formation in the presence of erythropoietin (Epo). *In vivo,* it causes an increase in granulocytes, monocytes, and eosinophils. It can activate these cells to efficiently fight microbes.

The G-CSF stimulates the formation of granulocyte colonies *in vitro.* It is able to act synergistically with interleukin-3 (IL-3), GM-CSF, and M-CSF. This cytokine is active *in vivo,* stimulating an increase of peripheral blood granulocytes.

The M-CSF stimulates the formation of macrophage colonies *in vitro.* It maintains the survival of differentiated macrophages and increases their anti-tumor activities and secretion of oxygen reduction products and plasminogen activators. This cytokine binds to a receptor that is the product of the protooncogene *c-fms.*

Interleukin-3 has multilineage stimulating activity and acts directly on the granulocyte-macrophage pathway, but also enhances the development of erythroid, megakaryocytic, and mast cells, and possibly T lymphocytes. In synergy with Epo, IL-3 stimulates the formation of early erythroid stem cells, promoting the formation of colonies of red cells in soft gel culture known as *BFU-E.* In addition, it supports the formation of early multilineage blast cells *in vitro.* IL-3 also induces leukemic blasts to enter the cell cycle and induces, either alone or in combination with other growth factors, the production of all the myeloid cells *in vivo.*

Stem cell factor (SCF) promotes survival, proliferation and differentiation of hematopoietic progenitor cells. It synergizes with other growth factors such as IL-3, GM-CSF, G-CSF, and Epo to support the colony growth of BFU-E, CFU-GM, and CFU-GEMM *in vitro.*
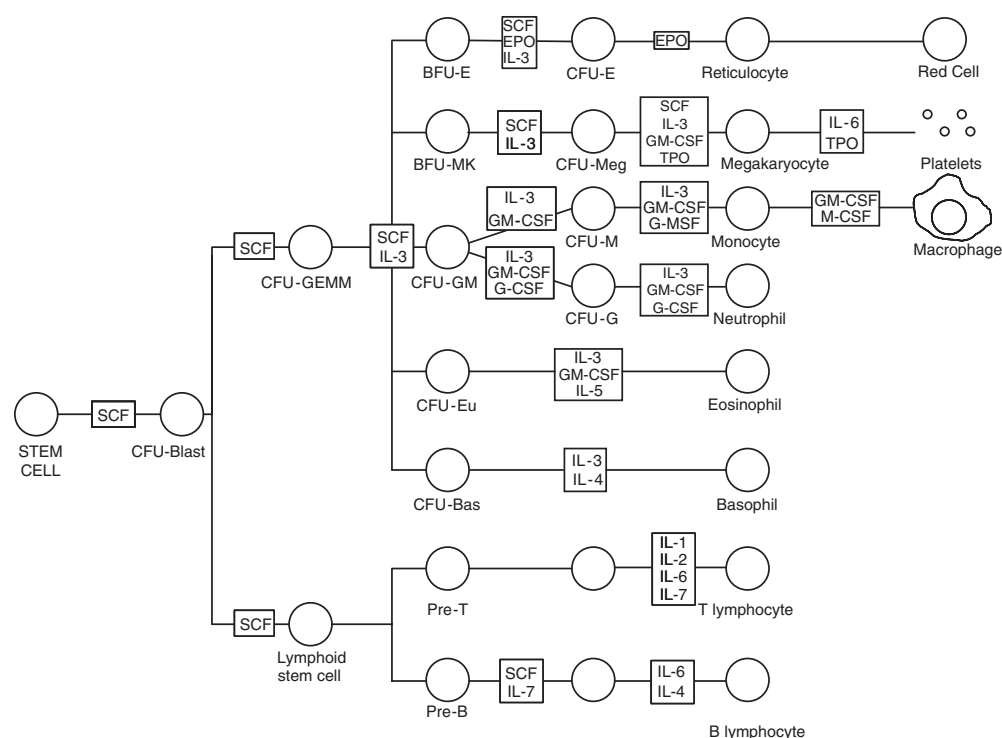
FIGURE 1   Scheme of hematopoiesis. The key progenitor cells and their growth factors are shown. CFU-Blast, colony-forming unit-blast; CFU-GEMM, CFU-granulocyte, erythrocyte, megakaryocyte, macrophage; BFU-E, burst-forming unit-erythroid; CFU-E, CFU-erythroid; BFU-MK, BFU-megakaryocyte; CFU-Meg, CFU-megakaryocyte; CFU-GM, CFU-granulocyte-monocyte; CFU-Eo, CFU-eosinophil; CFU-Bas, CFU-basophil; SCF, stem cell factor; IL-3, interleukin-3; GM-CSF, granulocyte-monocyte colony-stimulating factor; EPO, erythropoietin; TPO, thrombopoietin.

Although SCF alone has a modest effect on colony growth, in the presence of other cytokines SCF increases both the size and the number of these colonies. It is a ligand for the c-kit receptor, a tyrosine kinase receptor that is expressed in hematopoeitic progenitor cells.

The growth factor Epo stimulates the formation of erythroid colonies (CFU-E) *in vitro* and is the primary hormone of erythropoiesis in animals and humans *in vivo*. It binds to a specific receptor (Epo-R). Production of erythroblasts is hormonally regulated by a feedback mechanism mediated by the linear correlation between tissue oxygenation of Epo-producing cells in the kidney mediated by oxygen-carrying hemoglobin in red blood cells. Anemia causes tissue hypoxia, resulting in an increase of serum Epo levels.

II. VITAMIN D RECEPTORS IN BLOOD CELLS

The genomic actions of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] are mediated by the intracellular vitamin D receptor (VDR), which belongs to a large family of nuclear receptors [1]. VDR forms a heterodimer with the retinoid X receptor (RXR); this complex regulates expression of target genes by binding to vitamin D responsive elements (VDREs) in the promoter regions of their target genes [2]. The mechanism of action of $1,25(OH)_2D_3$ via the VDR is discussed in Chapters 11 and 13.

Expression of VDR has been detected in various normal and leukemic hematopoeitic cells. It is expressed constitutively in monocytes, in certain subsets of thymocytes, and after *in vitro* activation of B and T lymphocytes [3–5]. Expression of VDR is induced in the lymphocytes of patients with rheumatoid arthritis, in human tonsillar lymphocytes, and in pulmonary lymphocytes of patients with tuberculosis and sarcoidosis [6–8]. In addition, lymphocytes of patients with hereditary vitamin D–resistant rickets type II (HVDRR) have various alterations of the VDR [9]. Also, fewer receptors have been detected in the peripheral blood mononuclear cells of patients with X-linked hypophosphatemic rickets [10]. Examination of a large array of myeloid leukemia cell lines blocked at various stages of maturation showed that they all

expressed VDR, albeit at different levels [3]. Bone marrow-derived stromal cells express VDR and show a reduction of their proliferation that occurs after their exposure to 1,25(OH)₂D₃. Both T-helper and T-suppressor lymphocytes express similar concentrations of VDR. In particular, T lymphocytes express high levels of VDR mRNA, whereas resting B lymphocytes express either very low or nondetectable levels of VDR transcripts [3]. Nevertheless, 1,25(OH)₂D₃ inhibits the synthesis of immunoglobulins (Ig) by B lymphocytes *in vitro* [11,12]. This suppression, however, could be the result of the inhibition of T-helper activity [12]. Production of lymphokines, including IL-2, is markedly decreased by 1,25(OH)₂D₃ in activated T lymphocytes, and this could cause the suppression of Ig synthesis [13–16]. The effects of vitamin D on the immune system are discussed in Chapter 36.

Studies by us in VDR knockout (KO) mice indicated that expression of VDR is dispensable for normal myeloid development [17]. No difference in the numbers and percentages of red and white cells were found between VDR KO and wild-type (WT) mice. Committed myeloid stem cells from the bone marrow cultured in methylcellulose formed similar numbers of colonies when grown in the presence of either GM-CSF, G-CSF, M-CSF alone or in combination with IL-3. Furthermore, bone marrow cells from VDR KO and WT mice formed a similar number and percentage of granulocyte, macrophage, and granulocyte/macrophage mixed colonies when cultured in methylcellulose with GM-CSF and IL-3. Under these conditions, treatment with 1,25(OH)₂D₃ dramatically increased the percentage of macrophage colonies derived from WT but not VDR KO bone marrow cultures. This observation demonstrates the requirement of VDR expression for 1,25(OH)₂D₃–induction of bone marrow progenitors into monocytes/macrophages. The proportion of T- and B-cells were normal in the VDR KO mice. However, the antigen-stimulated spleen cells from VDR KO mice produced less IFNγ and more IL-4 than those from WT mice, indicating impaired Th1 differentiation. Additionally, IL-12 stimulation induced a weaker proliferative response in VDR KO splenocytes as compared to WT, and expression of STAT4 was reduced. These results suggest that VDR plays an important role in the Th1-type immune response.

The HL-60 myeloblastic cell line cultured in the presence of 1,25(OH)₂D₃ (10⁻⁷ *M)* has a 50% decrease of VDR protein levels at about 24 hr, which returned to normal levels after 72 hr; no change of VDR mRNA expression occurred [3]. These data suggested that one of the major sites of regulation of VDR expression occurs at the posttranscriptional level. The same cell line exposed to a lower dose of 1,25(OH)₂D₃ for 12 hr

appeared to have an increased number of VDRs, as determined by immunoprecipitation, which returned to normal levels after 72 hr [18].

The HL-60 myeloblasts cultured with retinoic acid (RA) and dimethyl sulfoxide (DMSO) or 12-O-tetra-decanoylphorbol-13-acetate (TPA) terminally differentiate into granulocytes or macrophages, respectively. The differentiation is associated with induction of high expression of VDR mRNA. Also, normal human nondividing macrophages express VDR mRNA, and these levels do not change after exposure to activating factors such as tumor necrosis factor α (TNFα).

The expression of VDR mRNA was not detectable in nonproliferating lymphocytes harvested from normal human peripheral blood, but VDR mRNA expression increased in proliferating lymphocytes after a 24 hr exposure to the lectin phytohemagglutinin-A (PHA), suggesting that in lymphocytes a major site of regulation of VDR expression is at the transcriptional level [3, 19]. Moreover, low levels of VDR expression were detected in low-grade non-Hodgkin's lymphoma (NHL) tumor samples and in the follicular lymphoma B-cell lines SU-DHL4 and SU-DHL5 [20].

The VDR can bind to the osteocalcin response element along with the activator protein-1 (AP1) complexes [21]. In addition, Jun and Fos proto-oncogenes are up-regulated by 1,25(OH)₂D₃ [22]. Jun-D DNA binding activity is increased during cell cycle arrest in the human chronic myelogenous leukemia RWLeu-4 cultured with 1,25(OH)₂D₃, suggesting that Jun D binding activity may play a role in the regulation of cell proliferation by 1,25(OH)₂D₃ [21].

III. EFFECTS OF VITAMIN D COMPOUNDS ON NORMAL HEMATOPOIESIS

The role of 1,25(OH)₂D₃ in cell differentiation was first described by Abe *et al.* [23] in the murine leukemia cell line Ml, which was induced to differentiate into more mature cells by 1,25(OH)₂D₃. Normal human bone marrow committed stem cells cultured in either soft agar with 1,25(OH)₂D₃ (10⁻⁷ *M)* or in liquid culture with 1,25(OH)₂D₃ (5 × 10⁻⁹ *M* for 5 days) and monocytes cultured in serum-free medium with 1,25(OH)₂D₃ (5 × 10⁻⁸ *M* for 7 days) differentiate into macrophages [24,25]. In further studies, these macrophages were functionally competent and able to release large amounts of TNFα and IL-6 [26]. Furthermore, the terminal differentiation of monocytes into mature macrophages can be obtained *in vitro* by culturing these cells in the presence of serum or in a serum-free medium with the addition of vitamin D₃ compounds [4,26,27].

As mentioned earlier, $1,25(OH)_2D_3$ is able to inhibit IL-2 synthesis and the proliferation of peripheral blood lymphocytes [12–15]. Indeed, $1,25(OH)_2D_3$ appears to be able to regulate many lymphokines. For example, Tobler *et al.* [28] showed that expression of the lymphokine GM-CSF is regulated by $1,25(OH)_2D_3$ through VDR by a process independent of IL-2 production. In particular, $1,25(OH)_2D_3$ was able to inhibit both GM-CSF mRNA and protein expression in PHA-activated normal human peripheral blood lymphocytes (PBL). The former occurred at least in part by destabilizing and shortening the half-life of the GM-CSF mRNA [28]. The down-regulation of GM-CSF was obtained at $1,25(OH)_2D_3$ concentrations similar to those reached *in vivo*, with a 50% reduction of GM-CSF activity occurring at 10^{-10} M $1,25(OH)_2D_3$. In addition, IL-2 did not affect the modulation of GM-CSF production by $1,25(OH)_2D_3$ in the PBL cocultured with $1,25(OH)_2D_3$ (10^{-10}–10^{-7} $M)$ and high concentrations of IL-2.

IV. EFFECTS OF VITAMIN D COMPOUNDS ON LEUKEMIC CELL LINES

All of the studies conducted so far with $1,25(OH)_2D_3$ emphasize the need for new vitamin D_3 analogs with greater anti-leukemic effects and less toxicity. In spite of the promising data obtained from *in vitro* studies, results of clinical trials in leukemia with $1,25(OH)_2D_3$ are limited in scope and thus far have exhibited only mediocre results. For example, the myelodysplastic syndrome (MDS) is associated with anemia, thrombocytopenia, and leukopenia and an increased number of myeloid progenitor cells in the bone marrow. Some patients with MDS go on to develop acute myeloid leukemia. We treated 18 MDS patients with increasing doses of $1,25(OH)_2D_3$ up to a maximum of 2 µg/day for 12 weeks. Although an improvement of at least one hematologic parameter occurred in 8 patients after more than 4 weeks, the response was not durable and not detectable at the end of the study at 12 weeks [24]. Nine patients developed hypercalcemia, which was the dose-limiting toxicity. In another study, seven MDS patients were treated with $1,25(OH)_2D_3$ (2.5 µg/day, for at least 8 weeks), with no beneficial effects [29].

A major drawback in using $1,25(OH)_2D_3$ is its calcemic effect, which prevents pharmacological doses of the compound from being given. Vitamin D analogs have been synthesized that have enhanced potency to inhibit proliferation and promote differentiation of cancer cells, with less calcemic activity as compared to $1,25(OH)_2D_3$ (see Chapters 80–88). Many of these analogs *in vitro* are between 10- and a 1000-fold more active than the parental $1,25(OH)_2D_3$ in their growth suppressive activity. A comparison of the relative anti-leukemic potencies of vitamin D compounds is provided in Table I. These analogs could provide

TABLE I Effect of Vitamin D Compounds on Clonal Proliferation of HL-60 Cells in Soft Agar and Calcium Levels in Mice

Compound	$ED_{50}{}^a$ ($\times 10^{-9}$ mol/l)	MTD^b (µg)	Reference
$1,25(OH)_2D_3$	4–18	0.0625	[102–109]
$1,25(OH)_2$-16-ene-D_3	0.015	0.125	[102]
$1,25(OH)_2$-16-ene-23-yne-D_3	3	2	[102, 104]
$1,25(OH)_2$-16-ene-5,6-trans-D_3	0.03	4	[103]
$1,25(OH)_2$-16-ene-24-oxo-D_3	0.2	ND^c	[107]
$1,25(OH)_2$-16-ene-19-nor-D_3	0.8	0.5	[107]
$1,25(OH)_2$-16-ene-24-oxo-19-nor-D_3	0.1	6	[106]
$1,25(OH)_2$-20-epi-D_3	0.006	0.00125	[104, 108, 109]
$1,25(OH)_2$-20-epi-22-oxa-24,26,27-trishomo-$D_3{}^d$	0.001	0.0125	[108]
$1,25(OH)_2$-diene-24,26,27-trihomo-$D_3{}^e$	0.23	0.25	[104]
19-nor-$1,25(OH)_2D_2{}^f$	2.4	0.1	[31]

^{a}ED$_{50}$ represents the effective dose achieving 50% growth inhibition of HL-60 cells.

^{b}MTD, Maximally tolerated dose; highest dose reported that did not produce hypercalcemia or other noticeable toxicities in mice when injected intraperitoneally, three times per week.

^{c}ND, not done.

^{d}Leo Pharmaceutical code name is KH 1060.

^{e}Leo Pharmaceutical code name is EB 1089.

^{f}Abbott Laboratories code name is Paricalcitol.

a larger therapeutic window for the treatment of hematologic malignancies, retaining the useful properties of 1,25(OH)$_2$D$_3$ [30].

A. Celluar Effects of Vitamin D Compounds on Leukemic Cells

The various vitamin D compounds have similar effects on inducing differentiation and inhibiting proliferation of several acute myeloid leukemia cell lines such as HL-60, U937, THP-1, HEL, and NB4. In contrast, more immature myeloid leukemia cell lines such as HL-60 blasts, KG1, KGla, and K562 do not respond to the hormone. Vitamin D analogs inhibit cell growth mainly by inducing cell cycle arrest. Many studies have shown that treated leukemic cell lines accumulate in the G0/G1 and G2/M phase of the cell cycle, with a concomitant decrease in the proportion of cells in S-phase [31–33]. These effects of vitamin D compounds on the cell cycle are discussed in Chapter 92. HL-60 cells treated with 1,25(OH)$_2$D$_3$ acquire the morphology and functional characteristics of macrophages. Expression of the cell-surface markers CD14 and CD11b are up-regulated. The cells become adherent to charged surfaces, develop pseudopodia, stain positively for nonspecific esterase (NSE) with a reduction of nitroblue tetrazolium (NET), and acquire the ability to phagocytose yeast during incubation with 1,25(OH)$_2$D$_3$ (10^{-10}–10^{-7} M for 7 days) [25,34]. In addition, the treated cells acquired the ability to degrade bone marrow matrix *in vitro,* raising the possibility that the cells may have acquired some osteoclast-like characteristics. Leukemic cells from acute myelogenous leukemia (AML) patients respond to vitamin D compounds when cultured *in vitro;* however, they are often less sensitive than the cell lines. They are often still able to undergo partial monocytic differentiation as assessed by NBT reduction, morphology, and phagocytic ability. Furthermore, their clonal growth is often inhibited [25,34]. The molecular targets of vitamin D$_3$ compounds in leukemic cells are described in the following section, and are summarized in Table II.

B. Molecular Mechanisms of Action of Vitamin D Compounds Against Leukemic Cells

Vitamin D compounds can exert their anti-cancer effects by activating the VDR and modulating the transcription of various target genes. Some of these target genes are associated with inhibition of growth and

TABLE II Molecular Targets of Vitamin D Compounds in Leukemic Cells[a]

Cell Cycle/Apoptosis	Oncogenes
Cyclin A ↑	c-myc ↓
Cyclin D1 ↑	Dek ↓
Cyclin E ↑	Fli ↓
p15 ↑	
p21^{Waf1} ↑	**Tumor Suppressors**
p27^{Kip1} ↑	PTEN ↑
Bcl-2 ↓	BTG ↑
Differentiation Markers	**Kinases**
CD11b ↑	PI3-K ↑activity
CD14 ↑	p38 MAPK ↑activity
	ERK 1/2 ↑activity
	PKC ↑levels

[a] Regulation of expression or activity may occur either directly or as a consequence of differentiation. See text for details.

induction of differentiation, but this modulation may not be a direct effect, as it may simply reflect the entire process of differentiation.

Myeloid leukemic cell lines treated with 1,25(OH)$_2$D$_3$ undergo an initial proliferative burst, which is followed by growth inhibition, terminal differentiation, and subsequent apoptosis [36,37]. Levels of cyclin A, D1, and E increased in U937 cells within 24 hours of 1,25(OH)$_2$D$_3$–treatment, decreasing after 48 hours, although cyclin dependent kinase (CDK) levels did not change [37]. The CDK inhibitors p21 and p27, important regulators of the cell cycle, were elevated during the periods of both proliferation and growth inhibition. A strong correlation appears to exist between early induction of p21 and the beginning of the differentiation program. The marked increase of p21 protein expression in response to 1,25(OH)$_2$D$_3$ may be due to enhanced posttranscriptional stabilization of p21 mRNA [38]. The up-regulation of p21 mRNA occurred independently of *de novo* protein synthesis, further supporting the hypothesis that p21 is an early response gene. Indeed, the p21 promoter contains a vitamin D response element, and induction requires the presence of VDR. Also, experiments using a variety of cell lines showed that 1,25(OH)$_2$D$_3$ and other differentiating agents could mediate their induction of p21-independent of an intact p53 gene [38].

Using differential hybridization, Liu *et al.* [39] showed that p21 is differentially expressed in response to 1,25(OH)$_2$D$_3$ in the myelomonocytic cell line U937. Transient overexpression of p21 and p27 in U937 cells

promoted the appearance of the cell surface differentiation molecules CD14 and CD11b. One series of experiments showed that the p15, p16, p18, p21, and p27 CDKIs were up-regulated in a time-dependent manner after the addition of $1,25(OH)_2D_3$ [39]. This induction occurred within 4 hr of the addition of $1,25(OH)_2D_3$ in the presence of cycloheximide (CHX), suggesting a direct transcriptional activation by VDR.

In another study, the protein expression of different G1-phase regulators has been examined in HL-60 cells exposed to different concentrations of $1,25(OH)_2D_3$. A strong up-regulation of p27 protein expression was evident after 72 hr of exposure to the compound, and it was dependent on $1,25(OH)_2D_3$ concentration. This up-regulation was also associated with increased levels of D- and E-cyclins, coinciding with the G1 arrest. These results suggested a prominent role of p27 in mediating the antiproliferative activity of $1,25(OH)_2D_3$ in this cell line [40].

Activation of the protooncogene c-myc by retroviral insertion or chromosomal rearrangement is a typical feature of human leukemias. The HL-60 leukemia cell line is characterized by high levels of expression of c-myc due to gene amplification [41, 42]. Treatment of this cell line with $1,25(OH)_2D_3$ results in a down-regulation of expression of this oncogene related to cell differentiation [43]. Supression of c-myc by $1,25(OH)_2D_3$ and its noncalcemic analogs has been demonstrated to occur at the transcriptional level in HL-60 cells [30,44]. $1,25(OH)_2D_3$ is thought to up-regulate proteins such as the homeobox gene, HoxB4, that binds to the first exon/intron border of c-myc to prevent transcriptional elongation, a process dependent on activation of PKCβ [45,46]. Another homeobox gene, HoxA10, was found by differential display to be a gene transcriptionally induced by $1,25(OH)_2D_3$ during differentiation of U937 cells [47].

$1,25(OH)_2D_3$ has a protective effect against apoptosis in HL-60 cells [48,49]. This effect lends support to the observation that monocytic differentiation interferes with programs leading to apoptotic death. In other cell types, inhibition of apoptosis correlates with elevated levels of Bcl-2, but this does not appear to be the case with myeloid cells. In fact, after culture with $1,25(OH)_2D_3$ a down-regulation of Bcl-2 was observed both at the mRNA and protein levels [49]. Exposure of HL-60 cells to $1,25(OH)_2D_3$ induces the expression of the protooncogene *c-fms,* which occurs in parallel with the induction of CD14 expression and a block of their cell cycle in G_0/G_1 phase [50]. In the chronic myelogenous leukemia (CML) cell line RWLeu-4, an inhibition of proliferation was observed after $1,25(OH)_2D_3$ treatment. Moreover, the binding activity of the protooncogene *junD* was enhanced by $1,25(OH)_2D_3$ in these cells

during their cell cycle arrest, whereas it was not decreased in a $1,25(OH)_2D_3$–resistant variant cell line [21].

Fusion proteins involving the retinoic acid receptor alpha (RAR α) with either the PML or PLZF nuclear proteins are the genetic markers of acute promyelocytic leukemias (APLs). Although APLs with PML-RARα are more sensitive to retinoic acid, expression of either PML-RARα or PLZF-RARα in U937 and HL-60 cells blocks terminal differentiation induced by $1,25(OH)_2D_3$ [51]. Both PML-RARα or PLZF-RARα can bind to VDR in U937 cells and sequester VDR away from its normal sites of localization [52]. Overexpression of VDR overcomes the block in $1,25(OH)_2D_3$–stimulated differentiation caused by the fusion proteins.

The cell lines HL-60 and U937 have been used to attempt to identify early response genes directly regulated by VDR. Bories *et al.* [53] identified a serine protease, myeloblastin, that was down-regulated by phorbol esters in promyelocytic cells, causing growth arrest and cell differentiation. They also isolated cDNAs coding for fructose 1,6-biphosphatase, whose expression is up-regulated by $1,25(OH)_2D_3$ in HL-60 cells and peripheral blood monocytes. Genes regulated during the course of $1,25(OH)_2D_3$–mediated HL-60 cell differentiation have been analyzed using cDNA array analysis [54]. Among the genes shown to be down-regulated were the putative oncogenes Dek and Fli-1, and up-regulated genes included the antiproliferative BTG1.

Increasing evidence suggests that both the antiproliferative and differentiation-inducing effects of vitamin D compounds require their modulation of the intracellular kinase pathways, p38 MAPK, ERK, and PI3-K. Activation of PI3-K has been shown to be required for $1,25(OH)_2D_3$–stimulated myeloid differentiation, as determined by induction of CD14 expression [55]. PI3-K was activated by $1,25(OH)_2D_3$ in THP-1 cells within 20 minutes. Pretreatment with the PI3-K inhibitors, LY 294004 and wortmanin, inhibited CD14 induction in response to $1,25(OH)_2D_3$ in THP-1 cells and peripheral blood monocytes. Furthermore, antisense oligonucleotides against PI3-K blocked induction of CD14 expression in THP-1 and U937 cells. Expression of the VDR was required for activation of PI3-K; and interestingly, VDR was found to associate with the active form of the kinase. Inhibitors of PI3-K have also been shown to block the differentiation induced by $1,25(OH)_2D_3$ in HL-60 cells [56].

Exposure of either HL-60 or NB-4 cells to differentiation-inducing concentrations of vitamin D compounds causes activation and nuclear translocation of MAPK [57–59]. In addition, the vitamin D_3 analog EB1089 was recently demonstrated to induce apoptosis of B-cell chronic lymphocytic leukemia cells from

patients, an event preceded by stimulation of p38 MAPK and suppression of ERK activity [60]. Furthermore, $1,25(OH)_2D_3$ was found to stimulate the transient [24–48h] phosphorylation of ERK1/2, which was followed by growth arrest and differentiation of HL-60 cells [61]. In another study, PD98059, an ERK1/2 inhibitor, blocked the $1,25(OH)_2D_3$–stimulated differentiation of HL-60 cells [62]. Activation of PKC by the phorbol diesters such as TPA, promotes monocyte differentiation of leukemic cell lines [63,64]. Differentiation of HL-60 cells in response to $1,25(OH)_2D_3$ is accompanied by increased levels of PKCβ, and this differentiation can be inhibited by the specific PKC inhibitor, chelerythrine chloride [65]. Other vitamin D analogs have been shown to stimulate expression and translocation of PKCα and delta during NB-4 monocytic differentiation [66].

Some of the effects of vitamin D compounds on the signaling pathways occur within seconds. For example, rapid changes in the phosphorylation status of MAPK (within 30 seconds) have been demonstrated in response to $1,25(OH)_2D_3$ in NB-4 cells [58]. These effects occur too quickly to be attributed to the genomic actions of vitamin D–mediated activated transcription of target genes by VDR. Nonetheless, $1,25(OH)_2D_3$–activated intracellular signaling pathways require the presence of VDR to stimulate monocyte/macrophage differentiation, as demonstrated by studies on bone marrow cells from VDR KO mice [17] and cells from patients with vitamin D–dependent rickets type II [67,68]. The rapid nongenomic activities of vitamin D are described in detail in Chapter 23.

C. Vitamin D Compounds in Combination with Other Agents

Because of the potential toxicity of $1,25(OH)_2D_3$ and its analogs at the concentrations required *in vivo*, various attempts have been made to use them with other compounds that might act synergistically to achieve an anti-leukemic effect capable of promoting cell differentiation, yet with an acceptable toxicity. A range of compounds with different mechanisms of action have been studied. Vitamin D compounds may cooperate with other differentiating agents such as retinoids, tissue plasminogen activator, and interferon (IFN). For example, $1,25(OH)_2D_3$ can potentiate IFN-γ action to induce the expression of CD11b and CD14. We and others have shown that the combination of vitamin D analogs and either all-trans-retinoic acid (ATRA) or 9-cis-retinoic acid (9-cis-RA) can potentiate the terminal differentiation process of HL-60 cells down the monocyte-macrophage pathway [69,70]. These findings have also been demonstrated in other studies [71,72].

Cells cultured in the presence of the combination of $1,25(OH)_2D_3$ and ATRA developed atypically, having a neutrophilic morphology, but in other properties were typical of monocytes (e.g., CD14 expression, ability to bind to bacterial LPS, and ability to develop sodium fluoride-inhibited NSE) [69,70]. The combination of ATRA (10^{-9} *M*) and the vitamin D_3 analogs $1,25(OH)_2$-16-ene-23-yne D_3 or $1,25(OH)_2$-23-yne D_3 (10^{-9} to 10^{-10} *M*) showed a synergistic effect on the induction of differentiation and inhibition of proliferation of HL-60 cells [73]. A decrease of *c-myc* expression was also observed in the presence of ATRA and $1,25(OH)_2$-16-ene-23-yne D_3. This down-regulation of *c-myc* was stronger than that observed using single agents and correlated with the initiation of differentiation. A synergistic antineoplastic effect of $1,25(OH)_2$-16-ene-23-yne D_3 and ATRA has been shown in HL-60 cells [73]. A HL-60 clone resistant to ATRA was much more sensitive to inhibition of proliferation by $1,25(OH)_2$-16-ene-23-yne D_3 as compared with $1,25(OH)_2D_3$. In addition, the induction of differentiation of these cells by $1,25(OH)_2$-16-ene-23-yne D_3 was much stronger in these cells in contrast to wild-type HL-60 cells. Another retinoid-resistant acute promyelocyte leukemia cell line (UF-1) was induced towards granulocye differentiation by $1,25(OH)_2D_3$, in association with stimulation of p21^{Waf1} and p27^{Kip1} expression [74]. These effects were enhanced by the addition of ATRA.

In the promyelocytic cell line NB4, carrying the translocation t(15;17) typical of APL, vitamin D compounds can act as weak inducers of monocytic differentiation [75,76]. Bathia *et al.* [77] showed that the combination of $1,25(OH)_2D_3$ and TPA resulted in a synergistic response in NB4 cells, causing a complete differentiation to fully functional adherent macrophages with a rapid arrest of cell growth in the first 24 hr. Remarkable inhibition of proliferation and induction of differentiation occurred when NB4 cells were cultured with both 9-cis-RA and KH1060 (a 20-epi-vitamin D_3 analog) [76]. ATRA and $1,25(OH)_2D_3$ also synergistically induce monocytic differentiation in the promonocytic cell line U937 [78]. The same group observed that U937 cells exposed to a moderate thermal stress responded with increased differentiation after the addition of $1,25(OH)_2D_3$ and ATRA [79].

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to enhance the differentiation of HL-60 cells in respnse to $1,25(OH)_2D_3$ and its analogs [80,81]. This effect may occur because of the ability of NSAIDs to inhibit an aldoketoreductase; this enzyme supresses the production of natural PPARγ ligands by blocking the conversion of prostaglandin D2 to prostaglandin J2 [82].

Vitamin D compounds have also been combined successfully with naturally occurring plant products. One of these is carnosic acid, a plant-derived polyphenol antioxidant recently shown to potentiate the prodifferentiative effects of $1,25(OH)_2D_3$ [83,84]. These studies demonstrated that carnosic acid enhanced the growth arrest and CD11b and CD14 expression induced by $1,25(OH)_2D_3$ in a HL-60 subline, and combining these compounds produced greater stimulation of VDR expression and activity as determined by gel-shift analysis. Differentiation was correlated with antioxidant activity, and was associated with activation of the RAF-ERK pathway and increased binding of the AP-1 transcription factor to the promoter of VDR. Furthermore, potentiation of differentiation by carnosic acid with $1,25(OH)_2D_3$ (10^{-9} M) did not lead to the increase in intracellular calcium concentrations as compared to when the cells were treated with 10^{-7} M $1,25(OH)_2D_3$ alone, although differentiation levels were equivalent.

Combining vitamin D compounds with traditional chemotherapy agents such as cisplatin, etoposide, and doxorubicin has been shown to reduce the concentrations required for their anti-leukemic activities [85, 86]. Studies in other cancers have also shown that a vitamin D compound combined with a chemotherapeutic agent was more effective than either agent alone [87–89]. A recent phase I clinical trial has demonstrated the therapeutic potential of this method, as very high doses of $1,25(OH)_2D_3$ could be given orally with paclitaxel [90]. Another novel approach to circumvent the calcemic side effects of vitamin D compounds has been to give high doses of $1,25(OH)_2D_3$ subcutaneously every other day [91]. In a phase I clinical trial, the maximum tolerated dose administered in this fashion was five times greater than the daily oral dose that had previously been shown to cause hypercalcemia [91].

V. VITAMIN D ANALOGS EFFECTIVE AGAINST LEUKEMIC CELLS

The first attempts using analogs focused on the compound 1α-hydroxyvitamin D_3 ($1\alpha OHD_3$), a vitamin D_3 analog that is efficiently converted to $1,25(OH)_2D_3$ *in vivo* by D_3-25-hydroxylase. This compound was administered to mice previously inoculated with the Ml leukemia cell line and showed greater activity than $1,25(OH)_2D_3$ [92]. Its conversion to the active form resulted in a more prolonged elevation of plasma levels of $1,25(OH)_2D_3$, and the dose (25 pmol, every other day) produced only a slight and not significant elevation of serum calcium. In addition, survival of the leukemic mice was increased by 50–60%; however, the more effective doses produced hypercalcemia. Also, the

administration of $1\alpha OHD_3$ produced tumor regression in follicular NHLs in rats, but hypercalcemia was the dose-limiting factor [20].

In one study, six patients with MDS were treated with $1\alpha OHD_3$ at 1 µg/day for a minimum of three months, but neither a good clinical response nor toxicity was observed in these cases [93]. In another clinical study, thirty MDS patients were included in two different groups: one group received 1α-OHD_3 at 4–6 µg/day and the other group received placebo; the patients were treated for a median of 17 weeks [94]. An improvement of hematologic parameters was detected in only one patient, but the authors felt the treated group had a greater proportion of patients who did not progress to leukemia as compared to the control group. Hypercalcemia and increased serum creatinine were observed in two patients, and these abnormal measurements regressed with reduction of the dose [94]. A case has been reported of an individual with chronic myelomonocytic leukemia (subtype of MDS) who achieved complete remission with 25-hydroxyvitamin D_3 therapy for 15 months; this remission was sustained for 15 months after the end of the treatment [95]. These results are surprising because $25OHD_3$ has low activity by itself and *in vitro* has little anti-leukemic activity. However, as substrate for 1α-hydroxylase it may lead to local production of $1,25(OH)_2D_3$ (see Chapter 79).

Calcipotriol (MC903) has a cyclopropyl group at the end of the side chain formed by the fusion of C-26 and C-27, a hydroxyl group at C-24, and a double bond at C-22. This compound was equipotent to $1,25(OH)_2D_3$ in inhibiting the proliferation and inducing the differentiation of the monoblastic cell line U937 [96,97]. In bone marrow cultures, the analog promoted the formation of multinucleated osteoclast-like cells, a vitamin D–mediated function. The effects of this compound on the immune system were very similar to those induced by $1,25(OH)_2D_3$. By interfering with T-helper cell activity, calcipotriol reduced immunoglobulin production and blocked the proliferation of thymocytes induced by IL-1 [98,99]. Exposure of the follicular NHL B-cell lines SU-DUL4 and SU-DUL5, carrying the t(14;18) translocation characteristic of the disease, to MC903 inhibited of proliferation only at high concentrations of the compound (10^{-7} M) [20]. At the same time, calcipotriol was 100-fold less active than $1,25(OH)_2D_3$ in inducing hypercalcemia and mobilizing bone calcium in rats [100]. However, the analog is rapidly inactivated in the intact animal, and therefore has been developed as a topical agent (see Chapter 101).

Introduction of a double bond at carbon 16 has proved to be an effective modification of $1,25(OH)_2D_3$ [101].

When combined with other motifs the 16 ene modification has led to a series of analogs with potent antiproliferative and differentiation-promoting activities with decreased calcemic effects. Prior studies by us have shown that vitamin D_3 analogs having the C-16-ene motif were almost 100-fold more potent than $1,25(OH)_2D_3$ in inhibiting growth of HL-60 leukemia cells, while the calcemic activity was the same or markedly less than $1,25(OH)_2D_3$ [102,103]. Combination of the C-16-double bond and the C-23-triple bond [$1,25(OH)_2$-16-ene-23-yne-D_3] produces a compound that is a more potent inducer of growth inhibition and differentiation in HL-60 cells than $1,25(OH)_2D_3$, and is 15 times less hypercalcemic in mice [102]. The analog $1,25(OH)_2$-16-ene-23-yne D_3 has potent antiproliferative and differentiating effects on leukemic cells *in vitro* [104]. In blocking HL-60 clonal growth, $1,25(OH)_2$-16-ene-23-yne D_3 has a potency about four times higher than $1,25(OH)_2D_3$. This compound administered to vitamin D–deficient chicks is about 30 times less effective than $1,25(OH)_2D_3$ in stimulating intestinal calcium absorption and about 50 times less effective in inducing bone calcium mobilization [97]. Further experiments have demonstrated the therapeutic potential of $1,25(OH)_2$-16-ene-23-yne D_3 by its ability to prolong markedly the survival of mice inoculated with the myeloid leukemic cell line WEHI 3BD+ when treated with a high dose (1.6 µg every other day) of the compound [105].

The $1,25(OH)_2$-16-ene-19-nor-24-oxo-D_3 was synthesized as a result of previous studies that isolated 24-oxo metabolites of potent vitamin D_3 analogs, which were formed in a rat kidney perfusion system [106]. We found that these 24-oxo-metabolites had markedly reduced calcemic activity, but possessed at least an equal ability as the unmetabolized analogs to inhibit the clonal growth of breast and prostate cancer cells and myeloid leukemia cells *in vitro*. Taken together, these findings prompted the chemical synthesis of a series of vitamin D_3 analogs with $1,25(OH)_2$-16-ene-19-nor-24-oxo-D_3 being one of the more exciting compounds, having the ability to inhibit acute myeloid leukemia cells in the concentration range of 10^{-10} M [107]. Remarkably, this compound had very little calcemic activity even when 6 µg was administered intraperitoneally to the mice three times a week [107].

The compound $1,25(OH)_2$-20-epi D_3 is characterized by an inverted stoichiometry at C-20 of the side chain. The monoblastic cell line U937 cultured with this compound showed a strong induction of differentiation [108]. It was also a potent modulator of cytokine-mediated T-lymphocyte activation and exerted calcemic effects comparable to $1,25(OH)_2D_3$ in rats. A study by us suggested that $1,25(OH)_2$-20-epi D_3 is the most potent vitamin D_3 compound at inhibiting the clonal growth of HL-60 cells and at inducing cell differentiation. In fact, it was about 2600-fold more potent than $1,25(OH)_2D_3$ in inhibiting the clonal growth of HL-60 cells and about 5000-fold more effective in preventing clonal growth of fresh human leukemic myeloid cells [109]. $1,25(OH)_2$-20-epi D_3 exerts its effects by binding directly to VDR as shown by a T-lymphocytic cell line established from a patient with vitamin D–dependent rickets type II (HVDRR) lacking a functional VDR. Clonal growth was not inhibited after treatment of these cells with high doses of either $1,25(OH)_2$-20-epi D_3 or $1,25(OH)_2D_3$ (10^{-7} M). In contrast, control experiments showed that these compounds [$1,25(OH)_2$-20-epi D_3 > $1,25(OH)_2D_3$] were powerful inhibitors of proliferation of a human T-cell leukemia virus type I (HTLV-I) transformed T-cell line that possessed VDR.

KH1060 is a potent vitamin D_3 20-epi analog with an oxygen in place of C-22 and three additional carbons in the side chain. It is about 14,000-fold more potent than $1,25(OH)_2D_3$ in inhibiting the clonal growth of the monoblastic cell line U937 [108]. It also has a powerful effect on other leukemic cells [70,108, 109]. However, it has the same hypercalcemic activity and the same receptor binding affinity as $1,25(OH)_2D_3$.

One promising new vitamin D analog is paricalcitol (19-nor-1,25-dihydroxyvitamin D_2), which has been approved by the Food and Drug Administration for the clinical treatment of secondary hyperparathyroidism. Clinical trials have demonstrated that it possesses very low calcemic activity [110,111]. Studies by us and another group have demonstrated that paricalcitol has antiproliferative, prodifferentiation activities against myeloid leukemia and myeloma cell lines [31,112]. Paricalcitol activity was dependent on the presence of VDR, as it was unable to induce differentiation of mononuclear bone marrow cells from VDR knockout mice, whereas cells from wild-type mice were differentiated towards monocytes/macrophages [31]. Furthermore, paricalcitol was able to inhibit tumor growth without causing hypercalcemia in nude mice. These observations have prompted us to begin a clinical trial of paricalcitol aimed at treating patients with MDS.

Potential mechanisms by which vitamin D analogs may have increased biological activity compared to $1,25(OH)_2D_3$ are: reduced affinity to the serum vitamin D–binding protein; decreased catabolism by 24-hydroxylase; retention of biological activities by metabolic products of vitamin D analogs; increased stability of the ligand-VDR complex; increased VDR DNA–binding and dimerization with RXR; and enhanced recruitment of the DRIP coactivator complex. These topics are covered in detail in Chapters 81–83.

In conclusion, new vitamin D analogs have potent anti-leukemic activity and lower hypercalcemic

effects than $1,25(OH)_2D_3$, and should be considered for the treatment of hematologic malignancies either alone or in combination with other differentiating agents. However, more phase I, II, and III trials are still necessary to assess the safety and effectiveness of these treatments.

VI. SUMMARY AND CONCLUSIONS

The hormone $1,25(OH)_2D_3$ plays a role in normal hematopoiesis, enhancing the activity of monocytes-macrophages and inhibiting cytokine production by T lymphocytes. It can also inhibit proliferation and induce differentiation of various myeloid leukemia cell lines. Its activity is mediated by vitamin D receptors that belong to the superfamily of steroid-thyroid receptors. However, the anti-leukemic activity of $1,25(OH)_2D_3$ *in vivo* is associated with high toxicity and the onset of hypercalcemia as the dose-limiting effect. Limited clinical trials have been performed for the treatment of preleukemia with differentiating agents including $1,25(OH)_2D_3$, but the *in vitro* effective dose caused hypercalcemia *in vivo*. Since the mid-1980s, many vitamin D analogs have been identified with reduced hypercalcemic activity and high potential to induce cell differentiation and to inhibit proliferation of leukemic cells. Further studies have been performed *in vitro* and *in vivo* using these analogs with other differentiating agents such as retinoids, in the hopes that the combination of agents working through different pathways could lead to synergistic activity. Proof of principle that $1,25(OH)_2D_3$ and its analogs are beneficial in cancer has occurred in experiments conducted *in vitro* and in laboratory animals; however, the results of currently ongoing and future clinical trials in patients using vitamin D analogs will determine their ultimate therapeutic value.

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Clinical Development of Calcitriol and Calcitriol Analogs in Oncology: Progress and Considerations for Future Development*

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I. INTRODUCTION

1,25 dihydroxyvitamin D_3 (calcitriol), a central factor in bone and mineral metabolism, is a potent antiproliferative agent in a wide variety of malignant cell types [1–12]. As noted in preceding chapters, calcitriol and calcitriol analogs have significant anti-tumor activity *in vitro* and *in vivo* in animal and human hematopoietic and epithelial cancer models. Calcitriol enhances the *in vitro* and *in vivo* anti-tumor effects of platinum and taxane analogs, as well as antimetabolites (cytosine arabinoside, gemcitabine), topoisomerase inhibitors (etoposide, irinotecan), and alkylating agents [12–14]. Calcitriol as a single agent induces G_0/G_1 arrest, modulates p27[Kipl] and p21[Waf1/Cipl] (the cyclin-dependent kinase (cdk) inhibitors implicated in G_1 arrest), induces cleavage of caspase 3, PARP, and the growth-promoting/prosurvival signaling molecule mitogen-activated protein kinase (MEK) in a caspase-dependent manner [4,9,11,12,15]. In association with these effects, full length MEK, phospho-Erk (P-Erk), and phospho-Akt (P-Akt) are lost. The phosphorylation and expression of Akt, a kinase regulating a second cell survival pathway, is also inhibited after treatment with calcitriol. In contrast to changes that occur during cytotoxic drug-induced apoptosis, the pro-apoptotic signaling molecule MEKK-1 is significantly up-regulated by calcitriol [9]. Enhancement of cytotoxic agent-mediated apoptosis by calcitriol is associated with an increase in PARP-, MEK-, MEKK-1, and caspase-cleavage; P-Erk and P-Akt decrease. In addition, the expression of the p53 homolog, p73, is strongly induced by calcitriol, and p73 can sensitize tumor cells to the cytotoxic effects of platinum and taxanes [20]. Glucocorticoids (GC) potentiate the anti-tumor effect of calcitriol and decrease calcitriol-induced hypercalcemia [16,17]. Both *in vitro* and *in vivo*, GC significantly increase vitamin D receptor (VDR) ligand-binding in the tumor while decreasing binding in intestinal mucosa [16], the site of calcium absorption [17]. P-Erk and P-Akt are decreased with calcitriol/GC, compared to either agent alone [16].

These preclinical data support the development of calcitriol-based approaches to cancer therapy. Historically, a limited number of trials in cancer patients have been completed testing vitamin D-based approaches.

While early studies were largely negative, the improved understanding of the molecular changes that occur following calcitriol treatment and new approaches to the use of calcitriol and analogs provide encouraging data on the potential to develop calcitriol-based anti-tumor approaches. This chapter will review the current status of the clinical development of vitamin D analogs in cancer therapeutics, emphasizing important new pharmacokinetic and combination therapy approaches.

II. CLINICAL TRIALS

A. Early Trials

Oral calcitriol and analogs such as EB 1089 and $1\alpha(OH)D_3$ have been used in a number of clinical trials. Hematopoietic disorders (myelodysplasia, acute leukemia) have been the most commonly studied diseases. These studies were largely negative and have suffered from a number of limitations: small numbers of patients, lack of control groups, and use of a calcitriol analog in combination with cytotoxic agents in single arm trials, such that conclusions regarding the contribution of the calcitriol analog are difficult [21–26]. Gross and colleagues and Osborn and co-workers conducted straightforward trials of oral daily calcitriol administration in prostate cancer [27,28]. Gross *et al*. studied patients in whom the prostate-specific antigen was rising following local therapy, and Osborn *et al*. studied men with androgen-independent prostate cancer. As noted in Chapter 94, both studies were carefully designed, with sufficient power to delineate positive effects of calcitriol. While both studies provided evidence suggesting some positive benefit of calcitriol, both studies illustrate the major shortcomings of all previous studies of calcitriol-based therapeutics: doses of calcitriol and the schedule employed were not reflective of calcitriol exposures achieved in preclinical models in which substantial anti-tumor effects have been demonstrated. Both Gross *et al*. and Osborn *et al*. utilized the daily doses of calcitriol employed in the management of benign disease (1.5–2.0 μg). In each study, perturbations of calcium metabolism occurred that led the investigators to limit dose escalation—hypercalciuria (Gross) and hypercalcemia (Osborn). These are the predicted toxicities of vitamin D–based therapies and might have led to investigators abandoning attempts to administer calcitriol in either epithelial or hematopoietic malignancies. Most approaches to overcoming the hypercalcemic effects of calcitriol-based therapies have focused on the development of "nonhypercalcemic" vitamin D analogs. Thousands of such analogs have been synthesized and as will be noted below, some are now entering clinical trials. However, two groups of investigators have evaluated a different approach to averting the hypercalcemic effects of vitamin D. Reflecting on the fact that *in vivo* and *in vitro* experiments utilize high exposure, limited duration treatment, Johnson and Trump as well as Beer and colleagues have evaluated the feasibility of administering high-dose, intermittent regimens of calcitriol.

B. High Dose, Intermittent Calcitriol Regimens

Smith and colleagues explored a higher dose subcutaneous regimen of calcitriol, hypothesizing that an every-other-day (QOD) schedule combined with a subcutaneous route of administration might permit safe dose escalation of calcitriol [29]. These investigators were able to administer 8 μg QOD calcitriol safely—this represents a >twofold dose escalation compared to the oral, daily schedule. Beer and colleagues studied oral weekly administration and showed that doses as high as 2.6 μg/kg (approximately 180 μg weekly) could be administered without toxicity. These workers also demonstrated that at doses of >0.5 μg/kg, there appeared to be loss of dose-proportional increase in systemic exposure as doses of oral calcitriol were increased. Importantly, no limiting toxicity was noted in the patients treated by Beer and colleagues. Muindi and co-workers further evaluated these findings of apparent "saturable absorption" during the conduct of a trial of paclitaxel (intravenous, weekly × 6) + dose escalation of oral calcitriol daily for three consecutive days each week (QD×3, weekly). Calcitriol was administered safely at doses as high as 38 μg QD×3 weekly in combination with paclitaxel [31]. These workers confirmed the loss of dose-proportional increase in calcitriol exposure with increasing dose. Loss of dose-proportional increase in exposure appeared to occur at 16–18 μg of calcitriol (Fig. 1). In this figure, baseline-subtracted serum calcitriol $AUC_{0\rightarrow 24hr}$ (area under the concentration-time curve for the 24-hour-period after calcitriol administration) is plotted against dose. A fit to the Michaelis Menten function ($AUC = a \times dose/(1 + b \times dose)$ indicates that $AUC_{0\rightarrow 24hr}$ is not proportional to dose ($a = 540 \pm 140$ pg.hr/ml.μg); if AUC were proportional to dose, b would equal 0. The effect of this nonlinearity over the range of doses studied is large; the value of $AUC_{0\rightarrow 24hr}$ at 38 μg is only 4 times that at 4 μg, instead of the 9.5 times expected for a proportional relationship. No deviation from linearity can be detected up to a dose of 17 μg (p=0.4). In this trial, patients with advanced cancer received paclitaxel (80 mg/m^2 weekly × 6) + escalating doses of calcitriol, QD×3 weekly×6. The starting dose of calcitriol was 4 μg po QD×3 weekly, and patients were entered
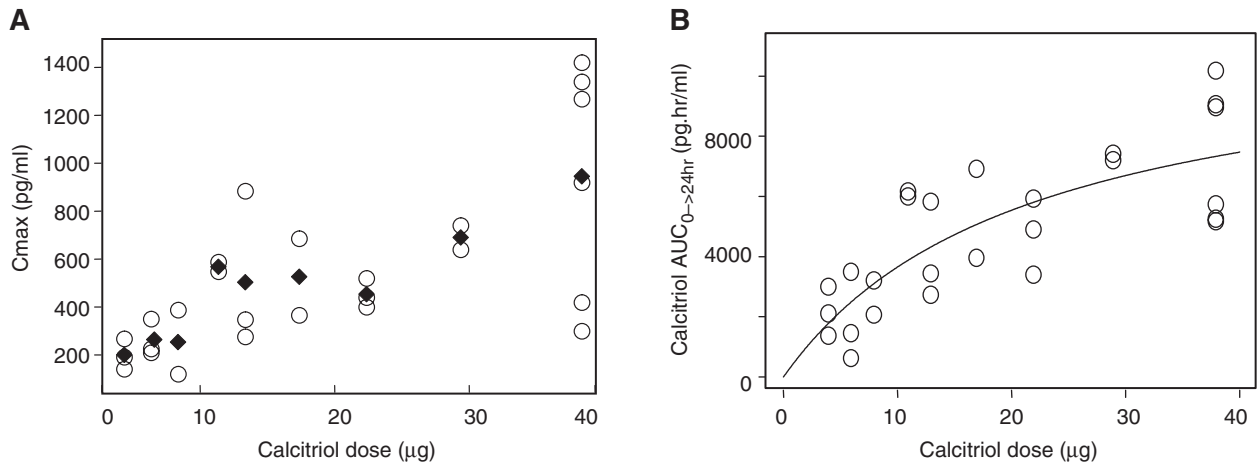
FIGURE 1 (**A**) Scatter plot of the maximum serum calcitriol concentration (Cmax) vs. calcitriol doses. Closed symbols represent mean values at each dose level. (**B**) Baseline-subtracted serum calcitriol $AUC_{0\rightarrow24hr}$ (area under the concentration-time curve for the 24-hour period after calcitriol administration) plotted against dose, a fit of the Michaelis-Menten function.

through the 38 µg dose level. No dose-limiting toxicity was encountered. In this study, the effect of calcitriol on paclitaxel pharmacokinetics was evaluated. No changes in peak concentration, AUC, or $t_{1/2}$ were noted, indicating the lack of drug-drug interactions between calcitriol and paclitaxel. In these studies as well as those of Beer and colleagues, the commercially available formulation of calcitriol (Rocaltrol®) was used—a formulation available only as 0.25 µg and 0.5 µg caplets. Hence, in these studies, patients were asked to take up to 75–100 caplets at one dose. To investigate whether this apparent limited absorption of calcitriol was related to pharmaceutical limitations posed by multiple caplet ingestion, Muindi and colleagues evaluated patients receiving escalating doses of calcitriol at 14 µg and higher using a liquid formulation of calcitriol (Fig. 2). No change in the curvilinear relationship between dose and AUC was noted.

Taken together these studies clearly indicate that high dose, intermittent administration of calcitriol is safe. Hypercalcemia was transient, and calcium returned to the normal range within approximately 24 hours after dosing. Table I summarizes the doses, concomitant drugs, and limiting toxicities seen in the series of trials conducted by these two groups of investigators. Dose escalation more than tenfold above that achieved with the daily doses of calcitriol is possible—without any apparent toxicity. It is also clear that pharmacokinetic considerations will complicate the use of the current standard formulations of calcitriol. Dose escalation in the studies of Trump and Johnson was ceased when it became clear that dose proportional increase in exposure was not feasible and substantial interpatient variation in exposure was noted (see Fig. 1).
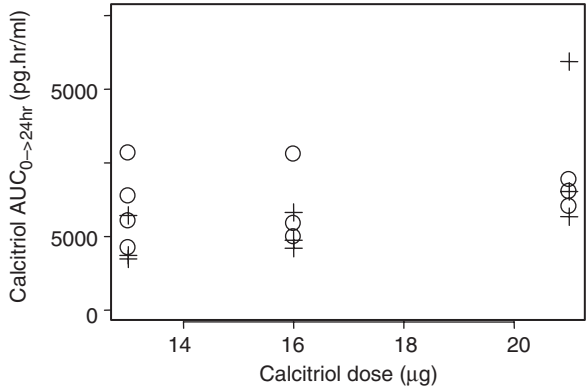

FIGURE 2 AUC from patients treated with calcitriol from the phase I trial of calcitriol where pk was determined on day one following calcitriol administration. Patients received either the capsule form (cross symbol) or the liquid form (open symbol).

TABLE I Clinical Experience with High Dose Oral Calcitriol

Route	Schedule	Agent[1]	MDA[2]	Hypercalcemia
Oral, oral	QD	0	2 µg	Yes
SQ, SQ	QOD	0	10 µg	Yes
Oral	QD×3	Dex	12 µg	No
Oral	QD×3	Carboplatin	24 µg	No
Oral	QD×3	Paclitaxel	38 µg	No
Oral	QD×3	0	24 µg	No
Oral	Weekly×1	0	2.6 µg/kg	No
Oral	Weekly×6	Docetaxel	0.5 µg/kg	No

[1] Agents administered with calcitriol.
[2] MDA, maximum daily dose administered.
QD, every day; QOD, every other day; QD×3, each day for 3 days/week.

III. LABORATORY-CLINICAL EXTRAPOLATIONS OF CALCITRIOL EXPOSURE

Among the first questions to arise in attempting to duplicate in the clinic the favorable anti-tumor *in vivo* effects of calcitriol that can be demonstrated in the laboratory is whether the systemic exposures obtained in the laboratory can be achieved in the clinic. While the initial bias of many has been that administration of high doses of calcitriol is not feasible, the studies of Beer and colleagues and Trump, Johnson, and Muindi clearly show that large doses of calcitriol can be administered safely—the limitation to date is pharmacokinetic, not toxicologic. While realizing that apparently saturable processes of absorption must still be overcome, Muindi and colleagues have characterized the systemic exposure achieved in mice at doses that exhibit anti-tumor and drug potentiating effects in several tumor models. These studies provide a rough "target concentration" that may be necessary to achieve in humans if these anti-tumor effects are to be realized. Figure 3 depicts the plasma concentration-time curve achieved in mice following intraperitoneal administration of "effective doses." As shown in Table II, at 0.125 µg (the lowest dose to consistently produce significant anti-tumor effects in mice), the $AUC_{0\rightarrow24hr}$ was 37.3 ng.hr/ml; this compares in man at a 38 µg dose to 7.5 ng.hr/ml. Similarly, in mice the Cmax was 9.2 ng/ml compared to 1.4 ng/ml in man. At the 0.042 µg dose in mice, an anti-tumor effect could be seen but was not consistently observed. Therefore, effective serum calcitriol levels are 5–7 times higher in mice than those achieved at the highest oral dose administered in man (38 µg) in the studies of Muindi and colleagues. The highest $AUC_{0\rightarrow48hr}$ reported by Beer and colleagues was 47 ng.hr/ml. Beer studied a relatively small number of patients at these high doses. Both groups' results are in agreement that intermittent high doses of calcitriol can be given safely and that attaining exposure in humans comparable to those required in mice to achieve optimal anti-tumor effects will be difficult with current formulations because they are inconvenient, highly
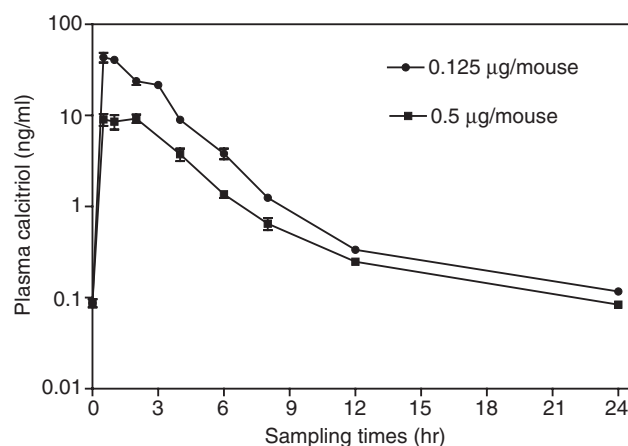

FIGURE 3   Plots of plasma 1,25-D_3 I concentration-time curves of normal C3H/HeJ mice; groups of 5–9 mice treated with a single 1,25-D_3 i.p. injection of either 0.125 or 0.5 mg dose/mouse. Plasma 1,25-D_3 concentrations were measured by RIA. Results are presented as mean ± SD.

variable in absorption, and at the highest doses display apparent saturable absorption characteristics.

IV. HIGH DOSE INTERMITTENT CALCITRIOL

Several other studies have confirmed that high-dose intermittent calcitriol is safe and well tolerated.

A. Calcitriol + Dexamethasone

Trump and colleagues [33] have completed a 43-patient study of calcitriol in escalating doses to a maximum of 12 µg calcitriol QD×3 each week together with dexamethasone (4 mg QD×4, weekly). While it was anticipated that this would be an aggressive calcitriol regimen that would be difficult to administer, this was not the case in practice. No patient ceased therapy because of hypercalcemia. Trimonthly urinary tract radiographs were completed to monitor for urinary

TABLE II   Calcitriol Pharmacokinetic Parameters Mice and Man

	Mouse (IP)			Man (PO)	
Dose (µg)	AUC (0→24) (ng.hr/ml)	Cmax (ng/ml)	Dose (µg)	AUC (0→24) (ng.hr/ml)	Cmax (ng/ml)
0.042	3.6	0.7	13	3.9 ± 1.4	0.5 ± 0.3
0.125	37.3	9.2	17	5.4 ± 2.1	0.5 ± 2.2
0.5	123.9	43.4	38	7.5 ± 2.1	1.4 ± 0.9

tract stones. Two patients developed stones; one symptomatic and one asymptomatic. Symptomatic improvement and PSA responses (>50% decline) were seen in 28% of patients, but this response frequency is not clearly greater than one might expect with dexamethasone alone.

B. Single Agent Calcitriol in Androgen-independent Prostate Cancer (AIPC)

Following this trial Trump and colleagues undertook a phase I dose escalation trial of calcitriol alone in AIPC to define the maximum tolerated dose and determine response. Dose escalation up to 36 µg QD ×3 weekly was possible without hypercalcemia or urinary tract stones. This study was terminated when it became clear that there was not dose-proportional increase in exposure as drug dose was increased.

C. Single Agent Calcitriol in Androgen-dependent Prostate Cancer

Beer and colleagues administered weekly oral calcitriol, 0.5 µg/kg, to 22 men with PSA rising after local therapy (prostatectomy or irradiation) [34]. No toxicity was encountered. No men met the criteria for response established by these investigators.

These data clearly indicate that very high intermittent oral doses of calcitriol can be administered safely. Single-agent oral calcitriol therapy (Rocaltrol®) appears to have limited activity, at least in prostate cancer, and optimal systemic exposure is limited by variable and incomplete oral absorption. It is important to emphasize that formal bioavailability studies of oral calcitriol have not been conducted to evaluate the exact mechanism of the loss of dose proportional increase in AUC with increasing dose. It is by inference and circumstantial evidence that this observation has been attributed to "decreased absorption." However, increased rate of metabolism consequent to increased calcitriol-induced 24-hydroxylase activity may also play a role.

V. CALCITRIOL + CYTOTOXIC AGENT COMBINATIONS

A. Carboplatin

Trump and colleagues initiated a phase I trial of carboplatin + calcitriol, based on the considerable data that

platinum analogs are potentiated by calcitriol [35–37]. Patients with advanced cancer were treated with carboplatin (AUC=5) every 28 days + escalating doses of calcitriol QD×3 every 28 days. Calcitriol starting dose was 4 µg QD×3. Studies were designed such that in each patient, carboplatin was given on day 1 before calcitriol in one of the first two cycles of treatment and on day 3 after two days of high dose calcitriol on the other. This permitted comparison of AUC of carboplatin in the same patient before and after pretreatment with calcitriol. Dose-limiting toxicity was not encountered in this trial. The AUC of carboplatin was higher in patients who received carboplatin following 3 days of calcitriol than in patients in whom carboplatin was administered before calcitriol (mean AUC=7.6 µg/ml.hr ± 1.8, carboplatin day 3 [DDDC] vs. AUC=6.6 µg/ml.hr ± 1.4, carboplatin day 1 [CDDD], $p=0.04$) (Fig. 4). While no-dose-limiting toxicity has been seen, myelosuppression (% change in platelet count) following the sequence carboplatin→calcitriol (CDDD) was less than that following calcitriol→carboplatin (DDDC), consistent with the change in AUC. No clinically detectable renal impairment was seen with either sequence. These data indicate that potentiation of carboplatin by calcitriol may in part be related to reduced carboplatin clearance. No patients became hypercalcemic. This trial was halted when the concerns regarding predictable and dose proportional exposure became evident in this and other studies of oral calcitriol (Rocaltrol®).

B. Taxanes

We have discussed the trial of Trump and colleagues evaluating the combination of high dose oral calcitriol + the taxane, paclitaxel. No dose-limiting toxicity was noted at calcitriol doses up to 38 µg QD×3 weekly. Recently, Beer and colleagues reported a phase I trial demonstrating that patients can tolerate weekly oral dosing of calcitriol at 0.5 µg/kg + docetaxel (36 mg/m² weekly×6) without significant toxicity [38]. This group has reported the results of a phase II trial of this regimen in men with androgen-independent prostate cancer. Among 37 men treated with weekly calcitriol (Rocaltrol®) + docetaxel, 81% (95% confidence interval [CI] 68–94%) achieved a PSA response rate as measured by a greater than 50% reduction in PSA [39]. This response rate appears greater than response rates of 38–46% reported in phase II studies of single-agent weekly docetaxel. Among 15 patients with measurable tumor masses 8 (53%, Ci 43%–75%) achieved a tumor mass response defined by standard criteria. These are quite encouraging data with respect to anti-tumor effects of calcitriol-based therapy and has led to an
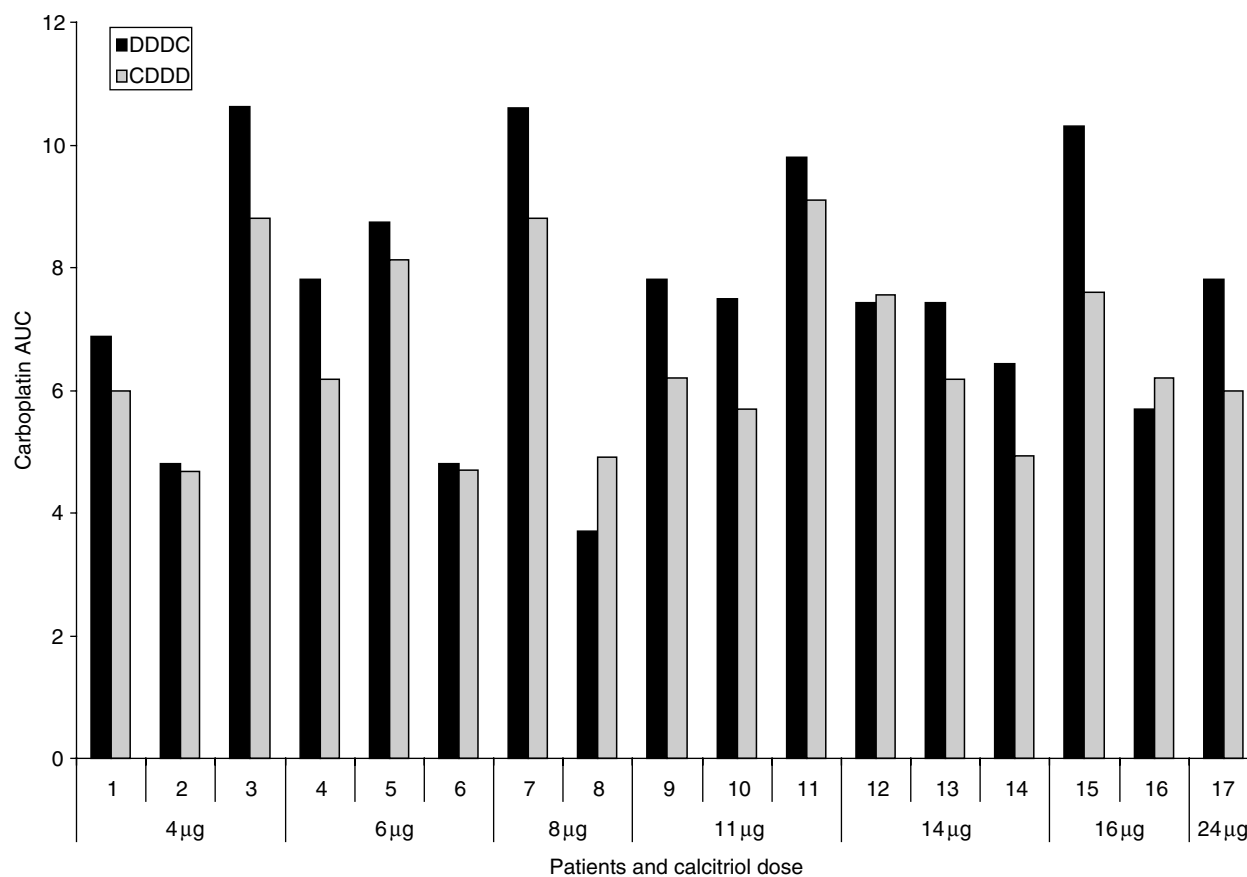
Figure 4　Carboplatin AUC from patients treated at selected doses of calcitriol with either carboplatin on day 1, calcitriol day 1, 2, and 3 (CDDD) (open bars), or calcitriol day 1, 2, and 3 followed by carboplatin on day 3 (DDDC) (closed bars). Carboplatin AUC is significantly greater with the DDDC sequence than the sequence CDDD ($p < 0.04$).

ongoing phase III trial to more rigorously evaluate this regimen (see below).

C. Reformulation of Calcitriol

In view of the fact that high-dose intermittent calcitriol is safe, feasible—but inconvenient and not dependably absorbed using the currently available and tested formulation Rocaltrol®—efforts have been undertaken to develop a more "bioavailable" and more convenient preparation of calcitriol. The express purpose of this effort is to develop a preparation that might allow full testing of calcitriol in high dose alone and with cytotoxic agents. A new formulation, DN 101 (Novocea, Inc.) has completed phase 1 testing; initial results indicate a more linear relationship between dose and exposure. No unusual toxicity or effects have been noted [40]. DN 101 is the calcitriol preparation being used in a randomized trial of docetaxel +/− calcitriol in AIPC. This trial is testing whether the increased PSA response rate seen in the trial of Beer *et al.* can be confirmed in a randomized trial.

VI. CALCITRIOL ANALOGS

While considerable work has been done to demonstrate that high-dose intermittent calcitriol administration is feasible and that blood levels in the range of those found to be effective in animal models have not yet been achieved, great interest remains in the development of vitamin D analogs that retain the antiproliferative and/or prodifferentiative properties of calcitriol with less propensity to cause hypercalcemia. Two analogs have been tested in substantial numbers of cancer patients: EB 1089 or seocalcitol and $1\alpha(OH)D_2$.

A. EB 1089 (seocalcitol)

Phase 1 trials of seocalcitol [(1(S),3(R)-dihydroxy-20(R)-(5′-ethyl-5′-hydroxy-hepta-1′(E),3′(E)-dien-1′-yl)-9,10-secopregna-5(Z),7(E),10(19)-triene] (see Chapter 84) have been conducted using a daily oral schedule of administration [41]. 7–15 µg/m²m is estimated to be tolerable and all patients who received 17 µg/sqm per day developed hypercalcemia. Preclinical data indicate

seocalcitol is 50–200 times more potent than calcitriol in terms of antiproliferative activity; these phase I data indicate that seocalcitol is 1/7–1/10 as potent in inducing hypercalcemia. As discussed elsewhere in this text, phase II studies have been conducted in breast, pancreatic, colorectal hepatocellular carcinomas (HCC), as well as leukemia [42,43] (Chapter 84). Anti-tumor responses have been seen in HCC, but not in the other diseases.

B. $1\alpha(OH)D_2$

This analog has been developed as one potentially more active and less prone to cause hypercalcemia. This agent is converted to $1,25(OH)_2D_2$ and $1,24(OH)_2D_2$, both of which activate VDR-mediated biologic effects; the $1,24(OH)_2D_2$ metabolite is substantially less potent than $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$ [44,45]. Liu and colleagues conducted a phase I trial of $1\alpha(OH)D_2$ in prostate cancer patients [46]. Daily dosing from 5 to 15 µg per day was employed. Hypercalcemia with dehydration and azotemia was noted at 15 µg QD; 12.5 µg QD was well tolerated in 3 of 3 patients. Two of 25 patients treated demonstrated evidence of anti-tumor effect; interestingly both responses were seen at "low" doses (5 µg and 7.5µg). It would appear that $1\alpha(OH)D_2$ is approximately $1/10$ as prone to induce hypercalcemia as a similar dose of calcitriol on a QD-dosing schedule.

The anti-tumor effects of seocalcitol and $1\alpha(OH)D_2$ are modest in the studies conducted. The activity of seocalcitol in HCC is of considerable interest in view of the importance of this disease worldwide.

VII. THE FUTURE

These data, in combination with the considerable preclinical information indicating the potential role of vitamin D–based therapies in cancer, continue to stimulate the interest of several research groups. The ongoing study of the new calcitriol formulation + docetaxel in AIPC will be very important in establishing the potential for calcitriol, on this dose and schedule, in the management of prostate cancer. Among the unanswered questions are: 1. *What is the proper dose and schedule?* Preclinical data indicate that all studies have been conducted at $1/5-1/10$ the drug exposure that is effective in animal models; and clinical evidence suggests that administration of the exposures effective in preclinical models may be safe and feasible. Trials of intravenous calcitriol and new formulations of calcitriol to establish the maximum possible dose on an intermittent schedule are underway. 2. *What is the*

"best analog"? While preclinical data suggest advantages for a number of analogs, substantial clinical work remains to evaluate the analogs currently in clinical trials. The greatest preclinical and clinical work has been done with calcitriol. 3. *Are there additional novel approaches that may capitalize on modulation of the vitamin D system?* Potentiation of the anti-tumor activity of growth factor receptor antagonists (gefitinib), as well as many other cytotoxics, has been well described in preclinical models [47–54]. Vitamin D analogs have been described to have antiangiogenic effects, as well as direct anti-tumor activities; this suggests that combinations and applications in settings where tumor blood vessels are the target may have merit [56–58]. Regional administration has merit. Regional arterial infusions (e.g. hepatic artery) or topical (e.g. cutaneous or bronchial) therapy are being investigated. Regional approaches have the great advantage of permitting the administration of high doses, locally with limited systemic effects. While definitive data regarding the use of vitamin D in the management of cancer remains elusive, preclinical data are persuasive and considerable progress has been made in developing clinical strategies utilizing vitamin D in the treatment of epithelial and hematopoietic cancers.

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Vitamin D$_3$: Autoimmunity and Immunosuppression

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I. INTRODUCTION

Since the mid-1980s, a variety of new properties and applications have been discovered for the hormone 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$]. Almost simultaneously the antiproliferative, prodifferentiating, and immunosuppressive activities of this metabolite of vitamin D were defined. It became obvious from the early investigations that in order to achieve maximal immunosuppressive activity *in vitro*, 1,25(OH)$_2$D$_3$ was required at a concentration higher than that needed to obtain antiproliferative activity. This observation explains in part the early success of the hormone when used for the treatment of psoriasis, whereas, currently, the inherent hypercalcemic properties of 1,25(OH)$_2$D$_3$ still prevent its clinical use for immunosuppression in humans. However, the development of analogs of the active metabolite has now broadened the potential clinical applications of the hormone. The optimal compound would be one that exerts maximal immunosuppressive activity while sparing the recipient of hypercalcemic complications. To achieve that goal, a variety of animal models of autoimmunity have been studied using 1,25(OH)$_2$D$_3$ and related analogs. This work has led to a potential application for the hormone in human autoimmune diseases. This review will concentrate on the mechanisms of action and effectiveness of 1,25(OH)$_2$D$_3$ in animal models of autoimmunity (excluding psoriasis, which is discussed in Chapter 101, and diabetes, which is discussed in Chapter 99) and describe the practical application of the hormone in humans for autoimmunity. Vitamin D regulation of the immune response is covered in Chapter 36.

II. AUTOIMMUNITY

A. Immune Mechanisms Operational in Autoimmunity

It is beyond the scope of this section to provide an extensive review of mechanisms involved in autoimmunity. However, by outlining some key factors described in the dysregulation of the immune system leading to the process, a better understanding of some targets of 1,25(OH)$_2$D$_3$ can be achieved.

The description of helper T-cells with different cytokine secretion patterns has opened a new understanding of cell–cell interaction and regulation. Three different T-helper cell subsets have been well described: Th0, Th1, and Th2 cells. The first subset, Th0, represents what appears to be an early precursor or transitory cell that subsequently differentiates into Th1 or Th2 cells on the appropriate stimulus, which is most likely provided by antigen-presenting cells (monocytes, macrophages, B-cells, or dendritic cells). One of these stimuli is interleukin-12 (IL-12), which can be produced by both monocytes, macrophages, and B-cells [1] and promotes Th0 cells to differentiate along the Th1 pathway [2]. The helper T-cell subsets Th1 and Th2 are determined by their cytokine secretion patterns. The Th0 cells produce an unrestricted pattern of cytokines. Th1 cells produce IL-2 and interferon-γ (IFN-γ), whereas Th2 cells produce IL-4, -5, -6, -10, and -13 [3]. Furthermore, Th1 cells can transfer delayed-type hypersensitivity (DTH) [4] and provide help to B-cells to produce the antibody isotype immunoglobulin G$_{2a}$ (IgG$_{2a}$), whereas Th2 cells help B-cells for IgGi and IgE secretion [5]. Because of their IFN-γ production, Th1 cells can also interact with macrophages to increase bactericidal properties [6]. These Th subsets also cross-regulate one another: IFN-γ produced by Th1 cells can down-modulate Th2 cells, and IL-4 and IL-10 produced by Th2 cells inhibits Th1 cells [7]. Interestingly, IL-10 produced by Th2 cells can indirectly inhibit Th1 cell responses by acting on monocytes that are required by Th1 cells for antigen-specific proliferation and lymphokine secretion [8], most likely by inhibiting IL-12 secretion [9]. The lymphokine IFN-γ produced by Th1 cells also enhances class II antigen expression [10].

This dichotomy between Th1 and Th2 cells has been confirmed in humans. A similar pattern of

cytokine secretion for both Th subsets is present [11]. Th1 cells express cytolytic activity against antigen-presenting cells and provide helper function for IgM, IgG, and IgA synthesis at low T-cell/B-cell ratios. At T/B ratios higher than 1:1, a decline in B-cell help is observed, related to the lytic activity of Th1 cells against autologous antigen-presenting B-cells [12]. This down-regulation of antibody responses could be operational *in vivo*. In contrast, Th2 cells develop in response to allergens or parasites, provide help for all immunoglobulin classes including IgE, and lack cytolytic potential [13]. The absence of lytic activity of Th2 cells may account for the long-term IgE responses of patients with atopy or parasitic infections [13]. High efficiency cloning of peripheral blood CD4+ T cells from healthy individuals generates the Th1, Th0, and Th2 cytokine profiles roughly distributed according to a 2:4:1 ratio [13]. The heterogeneity of the cytokine profile in humans is not restricted to CD4+ cells; CD8+ cells, which have the phenotype of cytotoxic and suppressor cells, can also be further defined by analysis of their lymphokine profile [14].

The recognition of the presence of regulatory T-cells leading to peripheral tolerance to extrinsic antigens or autoantigens has helped to understand some of the mechanisms involved in autoimmunity and to provide potential tools for new therapeutic agents [15]. Among those regulatory cells, CD25+T-cells appear to provide a protective effect in the prevention of the autoimmune process [16]. The role of Th2 cells as regulators of physiologic autoimmunity remains unclear. However, a protective effect of Th2 has been shown in situations of induction of autoimmunity such as antigen administration [15]. Further evidence of such a down-regulating role of Th2 cells has been provided by animal models: abrogation of tolerance in IL-4 deficient mice [17] or prevention of diabetes of non-obese diabetic (NOD) mice with administration of Th2 cytokines such as IL-4, IL-10 or IL-13 [18]. Induction of regulatory cells by T-cell vaccination such as T-cell receptor (TCR) peptide vaccination has been accomplished in experimental animal models and provides promising possibilities [19].

B. Mechanisms of Action of 1,25(OH)$_2$D$_3$ in Autoimmunity

A significant body of evidence suggests that while the resulting effects of 1,25(OH)$_2$D$_3$ on the immune response are suppressive, its actions are complex. The final response results in part from the interaction of the hormone with both antigen-presenting cells (APC) and T-cells leading to a dual response: suppression of

enhancers and amplification of down-regulators of the immune response.

1. HELPER T-CELLS

From the early discovery of the action of 1,25(OH)$_2$D$_3$ on the immune system, an interest sparked by the discovery of VDR in lymphocytes, a direct antiproliferative effect of the hormone on T-cells has been described [20]. However, its specificity of action resulted from the analysis of cytokine production by T-cells. The sterol inhibits the production of the Th1-mediated cytokines IL-2 and IFN-γ, both at transcriptional levels [21,22]. The pro-Th2 cytokine pathway appears to be spared by the sterol, but controversy has recently risen as to the net effect of the hormone on IL-4 production. First, 1,25(OH)$_2$D$_3$ leads to an increased production of IL-10 by helper T-cells [23]. By studying human helper T-cell clones, already committed to a specific antigen, the following observations were made after exposure of the cloned T-cells to the sterol. Helper T-cell clones were isolated from atopic patients sensitive to the rye grass antigen Lol pI and characterized as Th0, Th1, and Th2 based on their lymphokine secretion pattern [24]. The Th subsets were activated with Lol pI and antigen-presenting cells in the presence or absence of 1,25(OH)$_2$D$_3$ or the analog 1,25(OH)$_2$-16-ene D$_3$, and the effect of the vitamin D compounds on the lymphokine production was analyzed (Fig. 1). Both 1,25(OH)$_2$D$_3$ and l,25(OH)$_2$-16-ene D$_3$ suppressed the production of IFN-γ by Th1 cells in a dose-dependent manner, but these compounds had minimal effect on IL-4 production by Th2 cells and only at the highest concentrations tested. Interestingly, Th0 cells, producer of both cytokines, showed a profound reduction in IFN-γ in the presence of the vitamin D compounds, whereas IL-4 secretion was less inhibited, suggesting once again a pro-Th1 effect of the hormone and its analog [25].

Further evidence for an immunosuppressive effect of 1,25(OH)$_2$D$_3$ on a Th1-mediated biological activity was provided by the passive transfer of myelin basic protein (MBP)-specific Th1 clones. A characteristic of Th1 cells is their ability to transfer delayed-type hypersensitivity (DTH) [4]. MBP-reactive T-cell clones were activated with syngeneic spleen cells and antigen (MBP) in the presence or absence of 1,25(OH)$_2$D$_3$ before being washed and transferred to the footpads of naive mice. Swelling, as an index of DTH, was measured before and 18 hr after cell transfer using a pressure-sensitive caliper. A complete inhibition of the passive transfer of DTH was observed with 10 nM 1,25(OH)$_2$D$_3$ (Fig. 2) [26]. These results suggested that the hormone could directly interfere with functional activity of Th1 cells.
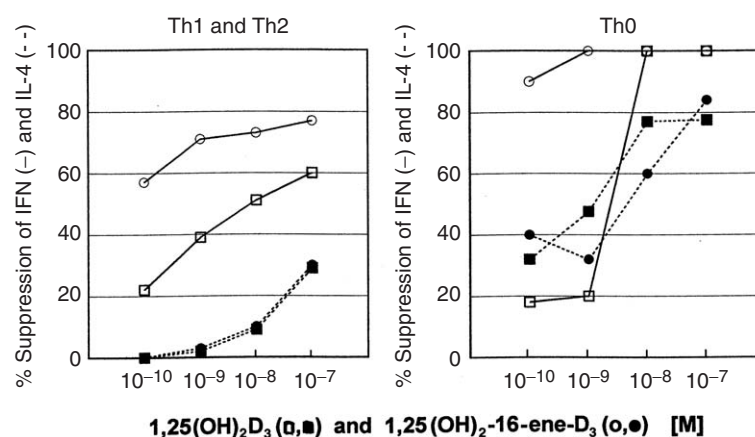
FIGURE 1   Effect of 1,25(OH)₂D₃ (○, ●) and analog 1,25(OH)₂-16-ene D₃ (□, ■) on lymphokine production by Th1 (left, open symbols), Th2 (left, filled symbols), and Th0 (right) cell clones. Th clones were activated with antigen, APC, and the lymphokines IL-4 and IFN-γ and assayed by radioimmunoassay. The data are expressed as percentage suppression.

More recently, it was shown that 1,25(OH)₂D₃ could directly affect naïve CD4+ T cells to enhance the development of Th2 cells [27]. When given *in vivo*, in IL-4–deficient mice, 1,25(OH)₂D₃ was less effective in its ability to suppress experimental autoimmune encephalitis (EAE) induction [28]. However, other studies have also shown a reduction of [29] or no effect on [30,31] IL-4 secretion by T-cells, questioning the 1,25(OH)₂D₃ "switch" to a pro-Th2 response. So far, these studies would suggest a primarily Th1–mediated inhibitory effect of 1,25(OH)₂D₃ with a resulting enhanced Th2 functional ability. Whether or not IL-4 is affected remains to be clarified in an *in vivo* situation.

2. SUPPRESSOR, CYTOXIC, AND REGULATORY T-CELLS

The modulation of suppressor/cytotoxic T-cells in the context of autoimmunity by 1,25(OH)₂D₃ is unclear. Studies in transplantation immunology have suggested the following. In the context of the mixed lymphocyte reaction [MLR] using human cells, when added at the initiation of the MLR, 1,25(OH)₂D₃ induced suppressor cell activity, reduced the generation of cytotoxic T-cells and the expression of class II antigen [32]. The steroid hormone could also inhibit natural killer cell activity [33–35] and reduce the activity of cytotoxic T cell lines [36].

Further alternative regulatory mechanisms can be attributed to the steroid hormone. 1,25(OH)₂D₃ could instead promote or allow for the development of regulatory T-cells and to induce tolerance. Naïve human and mouse CD4+T-cells can be induced into regulatory cells producing IL-10 by treatment with 1,25(OH)₂D₃ [23]. Transplantation tolerance through the induction of CD4+CD25+ regulatory T-cells can be achieved with 1,25(OH)₂D₃ [37]. Finally, the steroid hormone can interfere with apoptosis by the down-regulation of CD95L, a cell surface molecule not only activating apoptosis but also promoting Th1 activation through antigen-presenting cell maturation [38].
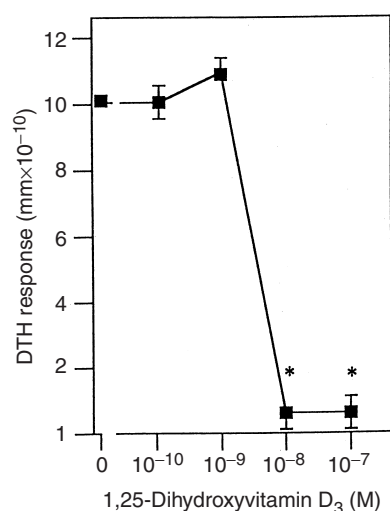

FIGURE 2   Delayed-type hypersensitivity (DTH) response in histocompatible recipients of cocultures of MBP-activated, cloned T cells and syngeneic spleen cells exposed to 20 μg/ml MBP. Cocultures were incubated for 5 days in the presence or absence of varying mean ± SD of the DTH response value in five individual mice. An asterisk signifies a significant ($p < 0.005$) decrease in the DTH response compared to cells not exposed to hormone. From Lemire and Adams [29] with permission.

3. ANTIGEN-PRESENTING CELLS

The first evidence of a direct effect of $1,25(OH)_2D_3$ on antigen-presenting cells was provided by the dose-dependent inhibition of IL-12, a pro-Th1 cytokine, by the steroid hormone [39]. Recent studies suggest that the resulting *in vitro* effect of the drug, when administered early, is to interfere with the differentiation and maturation of APC, thereby leading to altered T-cell responsiveness. *In vitro* treatment with $1,25(OH)_2D_3$ and analogs leads to down-regulated expression of the co-stimulatory molecules CD40, CD80, CD86 and to decreased IL-12 and enhanced IL-10 production, resulting in decreased T-cell activation, and with APC with tolerogenic properties [40]. Some *in vivo* evidence of such tolerogenic properties of APC induced by $1,25(OH)_2D_3$ and analogs has been shown in allograft rejection models with the sterol given orally [37] or following the passive transfer of $1,25(OH)_2D_3$ treated APC [41].

C. Effects of $1,25(OH)_2D_3$ on Animal Models of Autoimmunity

Table I illustrates the most representative studies of $1,25(OH)_2D_3$ and analogs in animal models of autoimmunity (excluding diabetes, Chapter 99). The first one, a long-standing useful model for the study of autoimmune diseases, has been the animal model of multiple sclerosis, EAE. In this model, immunization of susceptible mice or rats with central nervous system proteins will induce a progressive paralysis in the recipients within two weeks. Developments in peptide technology have led to a higher rate of disease induction in the susceptible recipients [42]. There is strong evidence that EAE is a Th1-mediated disease since antigen-specific Th1 cells can transfer disease [43]. Moreover, at the peak of the disease, there is a predominance of Th1 cytokines (IL-2 and IFN-γ) in the central nervous system of the mice; during remission, IL-10 prevails, suggesting a Th2 predominance [44]. In this model, $1,25(OH)_2D_3$ and analogs can prevent the induction and the relapses of the disease [31]. While $1,25(OH)_2D_3$ clearly exerts a Th1 inhibitory effect in favor of a pro-Th2 cytokine secreting effect *in vitro*, this dichotomy is harder to demonstrate *in vivo* [45–47].

A particularly interesting model is the experimental lupus of MRL/l mice. Lupus is an autoimmune disorder that leads to the formation and deposition of immune complexes throughout the body. Sites of predilection include the kidneys, causing nephritis, often with renal failure, and the skin, causing rash and inflammation. A potential role of Th1-mediated IgG_{2a} in the pathogenesis of the disease was suggested by

treatment of MRL/1 mice with anti-IgM anti-sera from birth. This resulted in a depletion of IgG_{2a} antibodies and prevented the development of skin, but not glomerular lesions [48]. A significant increase (eightfold) in IgG_{2a}-producing cells is observed in MRL/1 mice between two to five months of age [49]. In response to thymus-dependent antigens, the IgG subclass profiles in all systemic lupus erythematosus (SLE) mice differ from those of normals, with a predominance of IgG_{2b} and IgG_{2a} rather than IgG_1. In addition, sera of the majority of MRL/1 mice contain rheumatoid factors that react most strongly with IgG_{2a} [50], The dependence of IgG_{2a} secretion on Th1 cells [5], as well as class II expression secondary to IFN-γ secretion by Th1 cells, would suggest an important role for the Th1 cell subset in the pathogenesis of experimental SLE. The administration $1,25(OH)_2D_3$ from an early age in these mice could completely inhibit the development of skin lesions, characteristics of this animal model [51]. The development of nephritis and resulting proteinuria was not prevented with the same treatment. However, a subsequent study suggested that a diet with a normal to high calcium content (0.87%) administered to MRL/l mice undergoing similar therapy with $1,25(OH)_2D_3$ could even prevent the development of nephritis (proteinuria) [52].

The animal model of inflammatory bowel disease, experimental murine inflammatory bowel disease, provides additional information about the potential mechanisms of action of the sterol besides reducing the severity of the disease. IL-10 knockout mice, made vitamin D–deficient, develop a severe wasting syndrome; treatment with $1,25(OH)_2D_3$ improved symptoms and prevented the progression of existing disease [61]. These observations suggest once again that the primary target of $1,25(OH)_2D_3$ is through inhibition of Th1 cell activity rather than direct stimulation of the Th2 pathway.

D. Effects of $1,25(OH)_2D_3$ in Autoimmunity in Humans

The first and most studied application for $1,25(OH)_2D_3$ in an autoimmune disease in humans is psoriasis and the experience is reviewed in Chapter 101. Limited application of the hormone for other autoimmune disease in humans results from the intrinsic hypercalcemic properties of the hormone, restricting the therapeutic potential of $1,25(OH)_2D_3$. Two significant advances have held promising perspectives for the use of the steroid hormone in humans: the synergistic properties of $1,25(OH)_2D_3$ with known immunosuppressive agents, such as corticosteroids [67]

TABLE I   1,25 (OH)$_2$D$_3$ and Analogs in Autoimmunity

Organ	Model species	Vitamin D$_3$ or analog	Dose (µg/kg)[a]	Outcome, treated/ controls (measure)	Serum calcium (mg/dl)	Ref.
Nervous system	EAE/mouse	1,25(OH)$_2$D$_3$	5/2d	80%/5% (survival)	9.7	Lemire [53]
	EAE/mouse	1,25(OH)$_2$D$_3$-16-ene D$_3$	5/2d	1/4 (disease activity)	11.2	Lemire et al. [54]
	EAE/mouse	1,25(OH)$_2$-24-oxo-16-ene D$_3$	7.5/2d	1/4 (disease activity)	9.7	
	EAE/mouse	1,25(OH)$_2$D$_3$-16-ene-23-ene-26,27-hexafluroro D$_3$	2.5/2d	3.3/5 (disease activity)	8.7	Lemire et al. [55]
	EAE/mouse	MC1288	0.2/2d	25%/92.8% (disease incidence)	10.2	Lemire et al. [56]
	EAE/mouse	Ro 63-2023	240/2d	100%/50% (survival) 1.4/3.1 (relapses)	10.0	Mattner et al. [31]
Thyroid	Experimental auto-immune thyroiditis mouse	1,25(OH)$_2$D$_3$	0.2/d	50%/85.7% (histologic incidence)	N/A[b]	Fournier et al. [57]
Joint	Adjuvant arthritis/rat	1,25(OH)$_2$D$_3$	0.2/d	11.9/16.9 (arthritic score)	12.0	Boissier et al. [58]
	Murine Lyme arthritis	1,25(OH)$_2$D$_3$	1/d	0.2/0.23 cm (ankle size)	11.2	Cantorna et al. [59]
	Collagen-induced arthritis	1,25(OH)$_2$D$_3$	2.5/d	0%/100% (disease incidence)	7.9	Cantorna et al. [59]
	Collagen-induced arthritis	MC 1288	0.1/d	50%/100% (disease incidence)	2.6 mmol	Larsson [60]
Bowel	Experimental Murine Inflammatory Bowel Disease	1,25(OH)$_2$D$_3$	0.25/d	1.7/3.0 (histology score)	3 mmol	Cantorna [61]
Kidney	Heymann nephritis/rat	1,25(OH)$_2$D$_3$	0.5/d	80/210 (mg urinary protein/day)	11.8	Branisteau et al. [62]
		KH1060	0.5/d	210/210 (mg urinary protein/day)	11	
Kidney	Mercury chloride-induced nephritis/rat	1,25(OH)$_2$D$_3$	0.1/d	180/780 (mg urinary protein/day)	>14	Lillevang et al. [63]
		KH1060	0.3/d	<20/780 (mg urinary protein/day)	>14	
	Nephrotoxic serum nephritis/rat	1,25(OH)$_2$D$_3$	0.5/d	<50/300 (mg protein/day)	11	Hattori [64]
	Lupus nephritis/mouse	1,24R(OH)$_2$D$_3$	0.1/d	1.0/3.3 (dipstick)	N/A	Koizumi et al. [65]
	Lupus nephritis/mouse	OCT	0.002–0.1/d	10–40%/80% (proteinuria incidence)	10.4	Abe et al. [66]
	Lupus nephritis/mouse	1,25(OH)$_2$D$_3$	5/2d	<4/≥6 (urinary protein/creatinine ratio)	8	Lemire et al. [51]
	Lupus nephritis/mouse	1,25(OH)$_2$D$_3$	5–10/d	0–0.2/2–2.5 (severity score)	N/A	Deluca [52]
Skin	Lupus/MRL mouse	1,25(OH)$_2$D$_3$	5/2d	None/present (skin lesions)	8	Lemire et al. [51]

[a]d, daily; 2d, every second day.
[b]N/A: not available.

and cyclosporine [57,58,68,69], and the development of $1,25(OH)_2D_3$-analogs with reduced hypercalcemic activity. These exciting possibilities have yet to be applied in the treatment of autoimmune conditions.

The ideal application for $1,25(OH)_2D_3$ in humans is a disease in which a significant autoimmune component plays a role and no efficacious treatment is available. Systemic scleroderma might represent such a disease. While the cause of the disease is unknown, the pathogenesis is multifactorial [70]. An early immuno-logic trigger may lead to expansion of fibrogenic clones of tissue fibroblasts accompanying clinical expansion with early migration of CD4+ and CD8+ cells with a preponderance of CD4+ cells in the skin of affected patients [71,72]. These activated T-cells express HLA-DR molecules, IL-2 receptors, and increased CD4+ markers [73,74]. By precisely suppressing those immune mechanisms, $1,25(OH)_2D_3$ could play a role in modulating the disease.

A tolerability and feasibility study of calcitriol in the treatment of systemic sclerosis was done in 10 patients with a diagnosis of systemic sclerosis [75]. One patient withdrew within two weeks of the study secondary to drug intolerance. The 9 patients (M/F ratio of 2/7; age: 44 ± 14 years), with a disease duration of 6.9 ± 4.6 years, completed the six-month trial. After initial assessment and instructions to patients to follow a low calcium diet (800 mg/day), calcitriol was started at low dosage (0.25 or 0.5 mcg/day) and increased on a monthly basis to the maximum tolerable dosage, i.e. urinary calcium excretion of ≤ 350 mg/24 hours and/or serum calcium ≤ 10.8 mg/dl. Patients were assessed monthly with total skin score (Modified Rodnan Score, 17 body parts, scale 1–3), maximum oral aperture, and fist closure. Vitamin D metabolism was assessed at entry and at termination of the study and included: serum levels of $1,25(OH)_2D_3$, 25-OH-D, intact PTH, serum calcium, and urinary calcium excretion. Safety monitoring included: measurements of serum calcium and urinary calcium excretion within one week following any change in dosage of calcitriol; renal function assessed by clearance of creatinine (24-hour urine collection), and renal ultrasound (to exclude nephrocalcinosis or nephrolithiasis). This was an unblinded study without a placebo control, done primarily for safety reasons and to establish a dose for a placebo-controlled study.

The maximum tolerated dosage of calcitriol was 1.4 ± 0.5 µg/day (ranging from 0.5 to 2 µg/day). The mean intrapatient change (mean $\pm$ SE) of total skin score from baseline to end of study was -10.8 ± 3.3, a change of significant magnitude. Figure 3 illustrates the mean total skin score of the whole group per month during the course of the study.
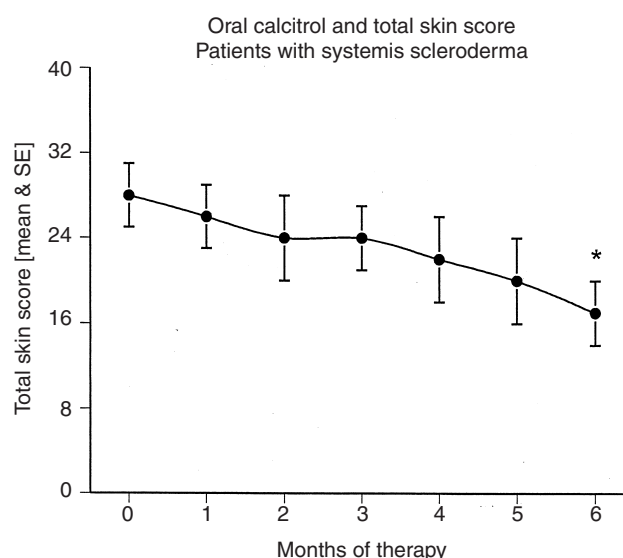

FIGURE 3   Individual total skin score (Modified Rodnan Score, 17 body parts, scale 1–3) of 9 patients with systemic sclerosis from entry to end of the 6-month treatment with calcitriol. Data are expressed as mean total skin score and standard error from the mean. *$p < 0.05$.

The most pronounced effect of calcitriol was observed on the change in the total skin score but some other physical variables improved while others did not change in therapy. While calcium excretion increased in all patients treated with calcitriol, no patient developed hypercalcemia, nephrocalcinosis, or nephrolithiasis. At the maximal tolerated dose of calcitriol, further reduction of calcium from the diet or awareness to comply to a strict diet improved the degree of hypercalciuria. Table II illustrates the biochemical and radiologic parameters studied.

TABLE II   Vitamin D Metabolism and Renal Function of Patients Treated with Calcitriol*

Parameters	Entry	Termination
Serum calcium (mg/dl)	9.3 ± 0.4	9.1 ± 0.37
Urinary calcium (mg/day)	112 ± 47	$270 \pm 134**$
Intact PTH (pg/ml)	26.9 ± 12.9	25.8 ± 27.2
$1,25(OH)_2D_3$ (pg/ml)	44.4 ± 14.2	$62.7 \pm 20.8**$
25(OH)D (ng/ml)	25.8 ± 10.4	25.1 ± 10.4
Renal function		
Serum creatinine (mg/dl)	0.6 ± 0.1	0.7 ± 0.2
Creatinine clearance (ml/min)	129 ± 47	115 ± 37
Renal ultrasound	normal (9/9)	normal (9/9)

*calcitriol administered at bedtime and blood drawn in the morning.
**$p < 0.001$.

TABLE III   Treatment of Scleroderma with 1,25(OH)$_2$D$_3$

Scleroderma	Patients/ treatment duration	Dosage	Outcome	Serum calcium (mg/dl)	Urine calcium[a] (ratio or mg/d)	Ref.
Localized	7/6 mo	0.25 µg/d × 1 wk	Decreased induration of slerotic lesions	N/A[b]	0.35	Elst [76]
		0.75 µg/d by 3 wks × 6 mo			(mean, end)	
Localized	7/2 to 24 mo	1.75 µg/d	Improved skin extensibility Clearing of lesions (4 pts)	N/A	N/A	Humbert *et al.* [77]
Localized and systemic	20 (localized) 7 (systemic)/ 9 mo	0.75 µg/d × 6 mo 1.25 µg/d × 3 mo	No difference	9.2	0.81 (mean, end)	Hulshof [78]
Systemic	11/6 to 36 mo	1.75 µg/d	Increased skin extensibility Improved oral aperture and flexion	<10.5	<300 mg/d	Humbert *et al.* [79]
Systemic	10/7 mo	0.5 to 0.75 µg/d	Decreased induration of slerotic lesions Joint mobility improvement	N/A	230 (mg/d) (mean, during treatment)	Hulshof [80]
Systemic	3/6 mo	0.5 to 0.75 µg/d	Decreased induration of slerotic lesions Increased skin extensibility Joint mobility improvement	10.8	100–400 (mg/d)	Caca [81]

[a]normal values.
ratio: <0.2.
24 hrs <400 mg/d.
[b]N/A: not available.

This study suggests a beneficial role of calcitriol in this human autoimmune disease. Such a role has been described so far in numerous studies of both localized and systemic scleroderma. Table III summarizes these results.

These results suggest that calcitriol can improve some of the manifestations of a severe autoimmune process when administered to patients. The calcium mobilizing properties of the hormone significantly restrict its optimal therapeutic potential since the dosages used are threefold higher than typically used for replacement. This disease, scleroderma, provides further indication that not all organ systems would be equally sensitive to the action of 1,25(OH)$_2$D$_3$, at least at the tolerated doses. For example, induration and elasticity of the skin is more susceptible to the positive action of calcitriol than internal organs (lungs, gastrointestinal tract, for example). It is therefore imperative that a double-blind, randomized, placebo-controlled trial of calcitriol in the treatment of systemic sclerosis be initiated to confirm a therapeutic application for 1,25(OH)$_2$D$_3$.

Then, when the development of analogs of 1,25(OH)$_2$D$_3$ with reduced hypercalcemic activity finally expands for other human application, the ultimate potential of the vitamin D compounds in the treatment of autoimmunity could be fully evaluated.

In conclusion, calcitriol exerts significant therapeutic as well as preventive actions in many animal models of autoimmunity. A potential role of calcitriol has emerged in some human autoimmune diseases, such as psoriasis (Chapter 101) and scleroderma. The development of fewer calcemic analogs of calcitriol for human application should expand its role in autoimmune diseases.

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Vitamin D and Diabetes

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I. INTRODUCTION

The influence of insulin on vitamin D and bone homeostasis is complex. Insulin enhances the 1α-hydroxylation of 25-hydroxyvitamin D *in vitro* [1], whereas insulin-like growth factor (IGF)-I also mediates the effects of hypophosphatemia on renal $1,25(OH)_2D_3$ production [2]. Diabetic rats with either spontaneous diabetes [3,4] or streptozotocin-induced diabetes [5–7] have decreased serum concentrations of $1,25(OH)_2D_3$.

Insulin-dependent diabetic children and also poorly controlled diabetic adults have decreased serum concentrations of $1,25(OH)_2D_3$ [8–11], whereas good diabetes control normalizes circulating $1,25(OH)_2D_3$ [9,12]. Most of the effects of insulin deficiency on serum $1,25(OH)_2D_3$ can be explained by decreased concentrations of the vitamin D–binding protein (DBP) [5,13]. Indeed, the DBP-corrected or calculated free concentrations of $1,25(OH)_2D_3$ are either normal or slightly increased in both diabetic animals and humans [13]. Kinetic studies in spontaneously diabetic BB (biobreeding) rats clearly showed increased metabolic clearance (>50%) of $1,25(OH)_2D_3$ but normal production rates. No such studies have been performed in human diabetics.

The classical vitamin D target tissues are, however, differently affected by insulin deficiency: the basal intestinal active calcium absorption and duodenal calbindin D concentration are decreased [3,14–16], but calbindin and intestal calcium absorption are still responsive to low calcium intake or $1,25(OH)_2D_3$ therapy [13]. The renal calbindin D-28K (CaBP) is not affected by insulin deficiency. At the bone level, a marked decrease in osteoblast number and function has repeatedly been observed in both diabetic animals and humans [17,18]. Therapy with even high doses of $1,25(OH)_2D_3$ clearly shows marked resistance of the osteoblasts from diabetics to $1,25(OH)_2D_3$ in contrast to the near normal responsiveness of the intestinal mucosa [13]. The osteoblast response, however, can be quickly normalized by insulin infusion [19], whereas IGF-I-therapy is partly effective both on $1,25(OH)_2D_3$ concentration [20] and osteoblast function [18].

Insulin and IGF-I thus have marked effects on the kinetics of $1,25(OH)_2D_3$ (production and catabolism) as well as on the sensitivity of classical target tissues (bone, intestine, kidney) to this secosteroid hormone. Indeed, Verhaeghe *et al.* showed in ovariectomized rats that rhIGF-I (250 µg/d) delivered sc for 28 days increased both plasma IGF-I and $1,25(OH)_2D_3$ concentrations, as well as plasma osteocalcin and urinary pyridinolines [21].

The present chapter will focus mainly on the effects of vitamin D and its activated form, $1,25(OH)_2D_3$, on the susceptibility for diabetes and its role in pathogenesis of the disease itself. Diabetes mellitus is a common disease in the Western world, with an estimated prevalence of 4 to 5%. The majority (95%) of the diabetic patients suffer from type 2 diabetes or noninsulin dependent diabetes, a metabolic syndrome characterized by insulin resistance and relatively inadequate insulin production by the β cell in the islets of Langerhans of the pancreas [22]. In this metabolic syndrome, it is still unclear whether the primary dysfunction is to be situated in the peripheral target organs of insulin (being mainly liver, fat, and muscle) or in the β cell [23]. Insulin resistance is induced by obesity and sedentary lifestyle and is also involved in the pathogenesis of cardiovascular disease. This insulin resistance is probably the major determinant of the disease, but β cell dysfunction is always present and will determine the severity of the clinical presentation. Type 1 diabetes, also known as juvenile or insulin dependent diabetes mellitus, is in etiology a totally different disease.

It has become clear in recent years that this disease is an autoimmune disorder, related to Graves' disease, characterized by a destruction of the insulin producing β cells in the pancreas by the body's own immune system [24]. Whereas type 2 diabetes is a typical disease of the obese and aging patient, type 1 diabetes mainly occurs in children and adolescents.

Since receptors for $1,25(OH)_2D_3$, the activated form of vitamin D, have been described in the main cells involved in the pathogenesis of both types of diabetes [25,26], scientific and clinical interest has focused on these molecules with respect to their potential role in the pathogenesis of the diseases, but even more with respect to their therapeutic potential in the prevention of the diseases [25,27,28].

The main effects of vitamin D and its activated form on the β cell, with direct implications for the pathogenesis and prevention of mainly type 2 but also type 1 diabetes, will be described and discussed. Furthermore, the role of vitamin D and $1,25(OH)_2D_3$ in the prevention of type 1 diabetes and its effects on the immune system of the animal models for the disease will be covered. Special attention is also given to the emerging evidence on correlations between vitamin D receptor (VDR) polymorphisms and risk for type 1 and type 2 diabetes. Older and novel data on the possible role of vitamin D deficiency in the pathogenesis of both diseases will be discussed.

II. VITAMIN D AND THE β CELL

A. Metabolic Effects

Since the early observations in 1980 [29] by Norman *et al.* that pancreatic insulin secretion is selectively inhibited by vitamin D deficiency, several reports have demonstrated an active role for vitamin D and especially its activated form, $1,25(OH)_2D_3$, in the regulation of the function of the endocrine pancreas, especially the β cell. Receptors for $1,25(OH)_2D_3$ have been described in β cells [30]. Not only does the classical $1,25(OH)_2D_3$ receptor seem to be present, but interesting observations also suggest the existence of a receptor localized in the membrane [30–35]. Apart from the vitamin D receptors, also the effector part of the vitamin D machinery is present: immune-reactive vitamin D–dependent CaBP has been demonstrated in β cells [36].

1. Effects of Vitamin D Deficiency *in Vivo*

The early observations mainly focused on the effects of vitamin D deficiency *in vivo* in several animal models and humans on insulin secretion and glucose tolerance [29,37–44]. Vitamin D deficiency clearly impairs insulin

(but not the other pancreatic hormones) secretion and induces glucose intolerance, while addition of vitamin D in the diet restores the abnormalities [31,37–42]. The effects of vitamin D deficiency and repletion on glucose homeostasis *in vivo* are, however, diverse and do not only involve the β cell. Rachitic animals do not have a normal food intake, lose weight, and cannot maintain a normal plasma calcium level. Moreover, these metabolic changes by themselves impair directly Ca^{2+} handling in the β cell and provoke β cell dysfunction and glucose intolerance [43,45].

Data from VDR knockout (VDR-KO) mice are conflicting, with some groups reporting impaired glucose tolerance [46], while in other reports no impairment of the glucose metabolism is reported [47]. Here, the background of the mouse in which the VDR-KO is introduced is of critical importance. Figure 1A shows a normal intraperitoneal glucose tolerance test (IPGTT) in VDR-KO mice in a C57Bl/6 background.

In the animal model of type 1 diabetes, the NOD (nonobese diabetic) mouse, vitamin D deficiency leads to impaired glucose tolerance, with decreased insulin secretion of the islets *in vitro* and earlier onset of diabetes *in vivo*, leading to a final increased diabetes incidence [Giulietti *et al*., unpublished data] [48]. In this model, however, the β cell as well as immune factors may contribute to the increase in diabetes incidence.

2. Effects of Vitamin D Metabolites *in Vitro*

The *in vivo* observations, where it is unclear whether the effects of vitamin D deficiency on glucose tolerance were direct effects of the deficiency on the β cell, were extended by *in vitro* data. Islets isolated from rachitic animals show impaired insulin release when cultured *in vitro* and challenged with glucose [49,50].

The abnormalities induced by vitamin D deficiency *in vivo* can be abolished by co-culturing the islets in the presence of high concentrations of $1,25(OH)_2D_3$ [31,49–51]. Even more convincing are the data on islets isolated from normal animals where most papers show a stimulation of insulin release upon glucose challenge in the presence of high doses of $1,25(OH)_2D_3$ [34,51,52]. Interpretation of the findings is sometimes obscured by the many different methods used (such as incubation time with $1,25(OH)_2D_3$, animal source of islets, type of glucose challenge), but overall an improved β cell function is observed in the presence of $1,25(OH)_2D_3$. Interestingly, some authors also studied the effects of $1,25(OH)_2D_3$ on insulin synthesis, and even this element contributing to normal β cell function is normalized or increased by $1,25(OH)_2D_3$ [37,53].

Progressively, insight is gained in the mechanism by which $1,25(OH)_2D_3$ might act on insulin secretion: a significant rise in cytosolic calcium levels is observed
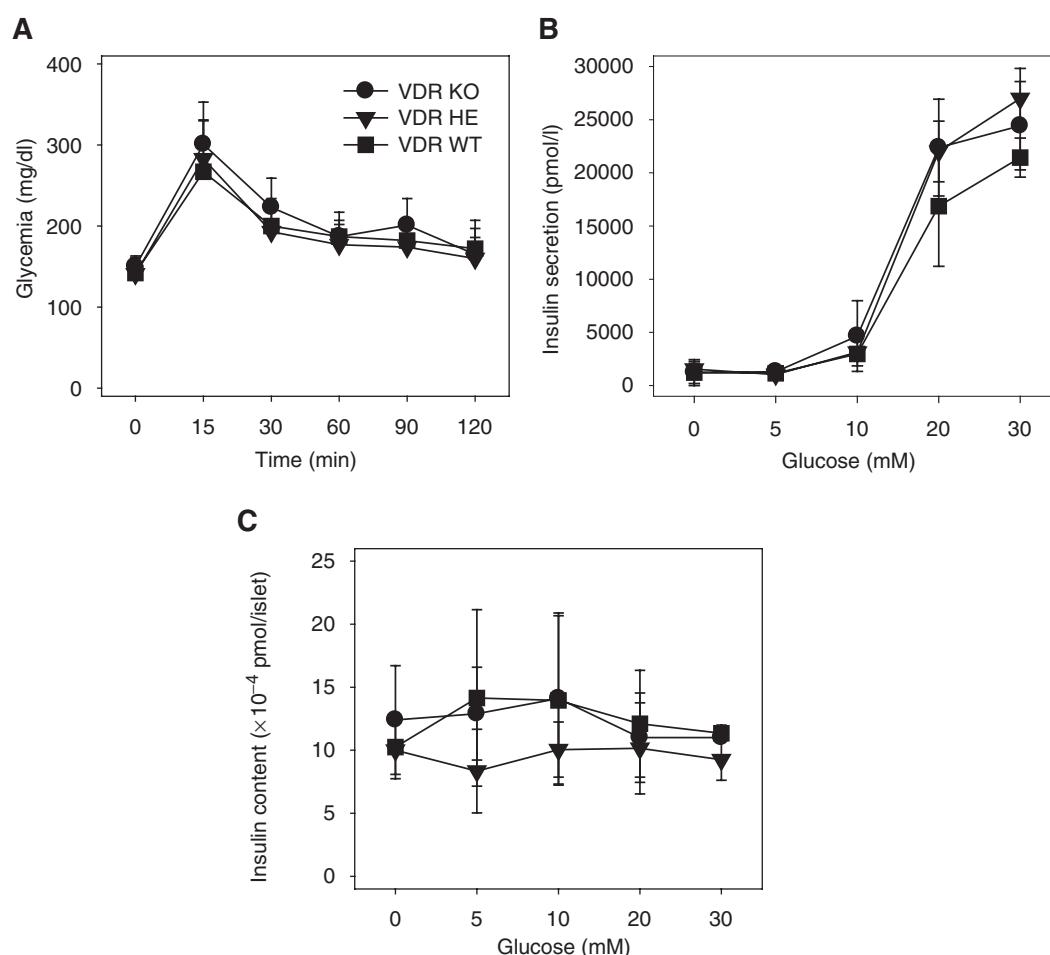
FIGURE 1   Intraperitoneal glucose tolerance test (**A**) in VDR KO, VDR (HE) and VDR WT mice. Glycemia was measured at different time points after glucose challenge. No difference in glycemia was noted between KO, HE and WT mice after glucose injection. (**B** & **C**) *In vitro* insulin secretion (**B**) and synthesis (**C**) of islets isolated from VDR KO, VDR HE, and VDR WT mice after incubation with different concentrations of glucose (0–30 mM). Again no differences were measured between VDR KO, HE and WT mice either for insulin secretion or insulin synthesis.

after incubation with 1,25(OH)$_2$D$_3$, resulting in insulin secretion. Controversy still exists on whether only an influx of extra-cellular calcium is responsible for this rise or whether also mobilization of intracellular calcium reserves is involved [30,34,49,50]. In the older papers, the genomic pathway was mainly studied and effects on calcium mobilization and insulin secretion were only observed after long incubation periods [49–52]. Recently, the non-genomic pathway, involving a putative plasmalemmal receptor inducing rapid calcium fluxes also has been implicated in the effects of vitamin D on insulin secretion [30–34].

The importance of calcium in this system is demonstrated by our own data in NOD mice that were vitamin D–deficient in early life, but maintained normal calcium levels. Here, isolated islets exposed to

glucose *in vitro* had perfectly normal insulin synthesis and secretion (Fig. 2A and B). In VDR-KO mice again the background on which the KO is introduced seems essential. Whereas some authors report abnormal insulin synthesis and secretion, VDR-KO mice on C57Bl/6 background have normal insulin synthesis and secretion (Fig. 1B and C).

3. EFFECTS OF VITAMIN D METABOLITES *IN VIVO*—CLINICAL IMPLICATIONS

Based on the observations *in vitro* and in animal models of vitamin D deficiency *in vivo*, clinical trials with vitamin D or 1,25(OH)$_2$D$_3$ on glucose metabolism have been performed. There are several reports demonstrating that vitamin D–depleted humans have reduced insulin secretion while, as expected, vitamin D treatment
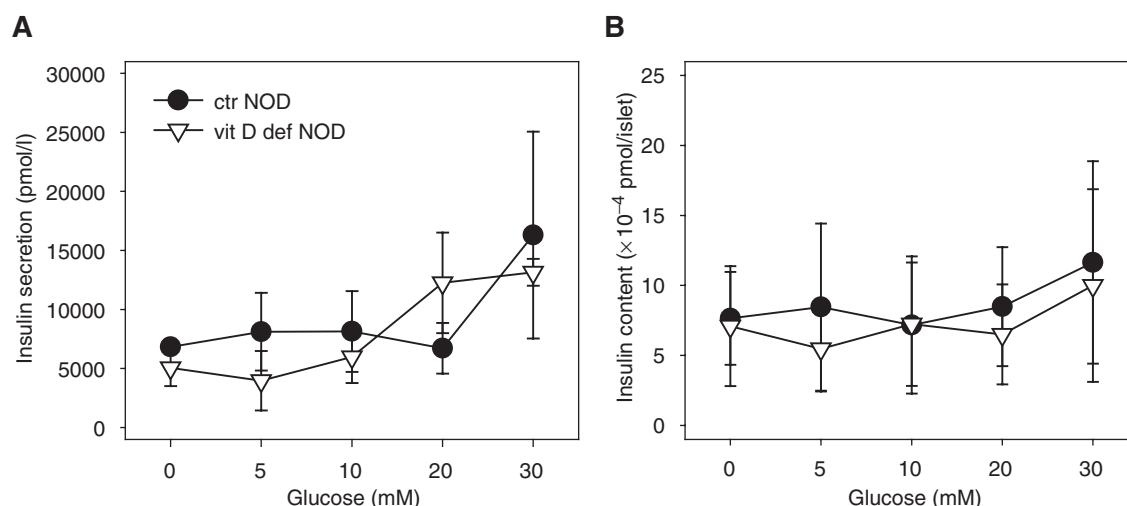
FIGURE 2   *In vitro* insulin secretion (**A**) and synthesis (**B**) of islets isolated from vitamin D–deficient NOD mice and control NOD mice. With the purpose of inducing vitamin D deficiency in NOD mice, 3-wk-old NOD mice were kept in UV-free surroundings and received a vitamin D–depleted diet containing 2% calcium and 1.25% phosphorus. Control mice were kept under similar conditions except their chow was supplemented with 2500 IU/kg vitamin D. From 100 days of age, all mice were fed control chow. No differences were measured between control and vitamin D–deficient mice either for insulin secretion or insulin synthesis.

(and calcium) reverses the vitamin D–deficient state and restores glucose tolerance [54]. However, the dose given and the administration route are often different between the studies. In vitamin D–depleted humans, $1,25(OH)_2D_3$ supplements of 2000 IU per day [55] or a single intramuscular injection of 100,000 IU [56] were administered, which makes comparison between the studies hazardous.

Interesting are the studies where the effects of $1,25(OH)_2D_3$ repletion in the relatively $1,25(OH)_2D_3$–deficient state of uremia were investigated [57]. In a study by Allegra *et al.*, uremic patients with low $1,25(OH)_2D_3$ serum levels were treated with 0.5µg of $1,25(OH)_2D_3$ (plus 500 mg of calcium) per day for 21 days, and IVGTTs were performed prior to and after treatment. Interestingly, $1,25(OH)_2D_3$ (plus 500 mg of calcium) caused an increment in glucose-induced insulin response only in the first few minutes of the stimulation (in uremia especially this rapid phase of insulin release is disturbed). In this study, however, repletion of $1,25(OH)_2D_3$ could not completely reverse the glucose intolerance. Orwoll *et al.* performed an interesting pilot study on possible clinical applications of $1,25(OH)_2D_3$ treatment in a situation of impaired insulin secretion without vitamin D deficiency [58]. Type 2 diabetic patients received $1,25(OH)_2D_3$ (1 µg/d for 4 days) in a double-blinded, placebo-controlled cross-over trial. Clear effects of $1,25(OH)_2D_3$ treatment were noted on parameters of calcium metabolism, but this study was unable to determine if hypovitaminosis D increases the risk of developing type 2 diabetes.

Since this could be due to the short duration of $1,25(OH)_2D_3$ treatment, this approach should not be discarded. Recently, another pilot trial showed beneficial effects of vitamin D supplements on first phase insulin secretion in type 2 diabetic women [59]. However, it was reported by Taylor and Wise that vitamin D supplementation in three cases of British Asians with vitamin D deficiency and type 2 diabetes led to increased insulin resistance and a deterioration of glycemic control [60].

In childhood diabetes, several epidemiological studies describe a correlation between a north-south gradient and the incidence of disease, as well as an inverse correlation between monthly hours of sunshine and the incidence of diabetes [61]. Also, a seasonal pattern of disease onset is well described in type 1 diabetes [62]. Dietary vitamin D supplementation is often recommended in pregnant women and children to prevent vitamin D deficiency. Besides the study of Stene *et al.*, who demonstrated that cod liver oil taken during pregnancy could reduce the risk for type 1 diabetes in their offspring (odds ratio = 0.30), the EURODIAB group suggested an association between vitamin D supplementation in infancy and a decreased risk for type 1 diabetes (odds ratio = 0.69) in a multicenter case-control study [63]. Moreover, Hypponen *et al.* reported that intake of 2000 IU of vitamin D during the first year of life diminished the risk of developing type 1 diabetes [64]. This study also confirmed that suspected rickets was associated with higher incidence for childhood diabetes (odds ratio = 2.6). Protection against

type 1 diabetes mellitus by vitamin D supplements may be due to a combination of immune effects and β-cell protection. In NOD mice or BB rats, no clear protection against type 1 diabetes can be seen by neonatal and early life supplements of regular vitamin D [65]. However, in NOD mice, clear preservation of insulin content in β-cells was observed.

B. Effects of Vitamin D on β Cell Characteristics

In the pathogenesis of type 1 diabetes, β-cell damage by cytokines and other inflammatory agents might play an important role. Sandler *et al.* demonstrated that $1,25(OH)_2D_3$ and some of its new structural analogs counteract the suppressive effects of IL-1β on β-cell function, such as insulin synthesis and insulin secretion [66]. Similar effects were observed in the case of IFNγ-induced impairment of β-cell function. Hahn *et al.* demonstrated that $1,25(OH)_2D_3$ and some of its analogs can prevent the IFNγ-induced decrease in insulin synthesis and secretion [53]. These effects were observed at high concentrations of $1,25(OH)_2D_3$ (5–10nM). On the other hand, Mauricio *et al.* could not demonstrate any effects of $1,25(OH)_2D_3$ on IL-1β induced β-cell dysfunction [67]. The main difference between the papers describing a protective effect and this latter study is the incubation time: in the former paper, incubation periods ranging between 48 and 72 hours were used, while in the latter study islets were only incubated with $1,25(OH)_2D_3$ for 24 hours. Considering observations made in similar conditions [49–52], this incubation timing might be the crucial point in the effect of $1,25(OH)_2D_3$.

Not only the effects of cytokines on β-cell function are altered by $1,25(OH)_2D_3$, but also the induction of surface markers by these cytokines appears to be blocked [53,68,69]. When neonatal rat islets were incubated with IFNγ, several surface markers such as MHC class II molecules and adhesion molecules (ICAM-I) were up-regulated as expected, Co-incubating the islets with $1,25(OH)_2D_3$ or one of its analogs decreased markedly the up-regulation of MHC class II molecules after IFNγ stimulation (15% MHC class II positive β-cells versus 30% in the presence of IFNγ only, $p < 0.001$). Similar results were obtained with an analog of $1,25(OH)_2D_3$, ZXY 1106 [53]. Riachy *et al.* demonstrated that incubation of human islet cells with IL-1β, IFNγ, and TNFα, decreased the insulin content after 48 hours of culture while leaving islet viability unchanged. However, 6 days after cytokine treatment, islet viability decreased to 60%. $1,25(OH)_2D_3$ treatment of human pancreatic islets incubated with IL-1β, IFNγ, and TNFα increased MHC class I expression, as well as nitric oxide (NO)

and IL-6 release [68]. Recently, this group published that vitamin D_3 was able to induce and maintain high levels of A20, an anti-apoptotic protein, in rat RINm5F cells and human islets after treatment with the three inflammatory cytokines [69].

These data showing β-cell protection against inflammatory agents involved in the pathogenesis of type 1 diabetes may have direct implications for the observed *in vivo* effects of $1,25(OH)_2D_3$ and its analogs in prevention of type 1 diabetes in animal models.

III. VITAMIN D AND THE IMMUNE SYSTEM IN TYPE 1 DIABETES MELLITUS

Prevention of juvenile diabetes is one of the major goals in health care for the future. When strategies for prevention of a disease are to be developed, a first requisite is the knowledge of the pathogenesis of the disease. From studies in two animal models for type 1 diabetes, the NOD mouse and the BB rat, but also in humans, it has become clear that type 1 diabetes can be considered an autoimmune disease, where a central role in the destruction of the β-cell is played by the body's own immune system. In this immune system action, almost all cells (monocytes/macrophages, T lymphocytes, B lymphocytes, NK cells, dendritic cells) play a role.

Up to now, most prevention studies have been carried out in the NOD mouse and can be divided into several major categories: pure immune suppression, immune modulation, antigen-(specific) tolerance induction, and β-cell protection. Results in NOD mice are promising for many of these treatments, but many obstacles to human applications still exist. All studies involving long-term immune suppression are inconceivable as strategy for the prevention of a chronic disease striking mainly children, and moreover, the preliminary results of these drugs in recent onset diabetic patients are disappointing. At this moment, a major interest is focused on immune modulation and β-cell protection, two characteristics of $1,25(OH)_2D_3$ and many of its newer analogs.

Since $1,25(OH)_2D_3$ can be produced by monocytes/macrophages/dendritic cells, and since receptors are present in several immune cells, a physiological role for this substance as a messenger or cytokine-like molecule between cells of the immune system is probable and therapeutic possibilities in the prevention of this autoimmune disease are to be expected. Moreover, as described above, clear β-cell protective effects of $1,25(OH)_2D_3$ against several inflammatory agents involved in β-cell destruction have been observed.

A. Primary Prevention of Type 1 Diabetes in Animal Models of Type 1 Diabetes by 1,25(OH)₂D₃ and Its Analogs

1. Early Intervention—Effects of 1,25(OH)₂D₃ on Diabetes Prevention

Two good animal models for type 1 diabetes in humans have been described up to now: the BB rat and the NOD mouse. Both strains develop a disease quite similar to human diabetes in a spontaneous manner, but the BB rat is characterized by severe immune abnormalities, and therefore most studies on pathogenesis and the development of therapeutic strategies have been performed in the NOD mouse. The NOD mouse was derived from a cataract developing substrain of the out-bred JcI-ICR mouse by selective inbreeding from 1974 to 1980 [70]. Diabetes develops spontaneously between the 12th and the 30th week of age, proceeded by the histological lesion of the disease, called *insulitis*. Insulitis is the reflection of the infiltration of the islets of Langerhans by a mixture of immune cells.

Chronic administration of pharmacological doses of 1,25(OH)₂D₃ can reduce the incidence of both insulitis and diabetes in NOD mice [71,72] (Table I). When, 1,25(OH)₂D₃ was administered at a dose of 5 μg per kg body weight every two days from the age of weaning (21 days) until the end of the life of the NOD mice (200 days), insulitis, the histopathological lesion of type 1 diabetes, was reduced from 80% in control mice to 58% ($p < 0.05$) in 1,25(OH)₂D₃–treated mice. Diabetes itself was reduced from 56% to 8% ($p < 0.001$). Although treatment was globally well tolerated, hypercalcemia and bone decalcification were seen. These data were confirmed by other groups [48]. Extensive immunological screening, including FACS analysis of major lymphocyte subsets, evaluation of T-cell proliferation (after CD3 or Con A stimulation), and NK-cell function, was unable to discover major changes in the treated versus control mice. A major finding was the restoration of a well-known defect of the

NOD mouse: the absence of suppressor cell function. The restoration of suppressor cell function could be demonstrated both *in vitro* and *in vivo* [72,73].

Adorini *et al.* demonstrated that the nature of the regulator cell induced by 1,25(OH)₂D₃ or its analogs is most likely a CD4+CD25+ cell [74,75].

The question remains however whether this restoration of suppressor cells is the main mechanism involved in protection against diabetes by 1,25(OH)₂D₃, since not only was there protection against diabetes, but also against insulitis, pointing towards interference with the induction of auto-immunity itself. Diabetes occurring in NOD mice after cyclophosphamide administration is believed to be due to an elimination of regulator cells [76,77]. The time needed for recovery of the immune cells after injection of a high dose of cyclophosphamide is different between various T-cell populations. Long-lived effector T-cells recover more promptly than the short-lived suppressor or regulator cells [78]. Several potential preventive therapies for diabetes have already been tested in this cyclophosphamide model. Protection against diabetes by therapeutic interventions that are believed to induce suppressor T-cells but that have no effect on autoimmune effector cells can be broken by cyclophosphamide. This was shown for the protection against diabetes achieved by Complete Freund's Adjuvant (CFA) or for the prevention of recurrence of diabetes in syngeneic islet grafts by BCG [79,80]. On the other hand, cyclophosphamide could not reverse the protection against diabetes in animals treated with the streptococcal preparation, OK432, as this substance prevents diabetes by inhibiting the generation of effector cells for β cell destruction [81]. Casteels *et al.* have demonstrated that 1,25(OH)₂D₃ can also prevent diabetes induced by cyclophosphamide in the NOD mouse [82].

Cyclophosphamide induced diabetes (78%) and insulitis (100%) in control animals. When 1,25(OH)₂D₃ was administered from day 21 until day 69, protection against diabetes (17%, $p < 0.01$) and insulitis (42%, $p < 0.005$) was observed. This protection was achieved

TABLE I   1,25(OH)₂D₃ and its Analogs in Animal Models of Diabetes and Islet Transplantation

Animal models of diabetes	Major effects	References
Diabetes		
type 1	Prevention of disease, inhibition of insulitis and diabetes	[71,72,82,104,133]
low-dose streptozotocin-induced	Decreased incidence of insulitis and diabetes	[88]
Islet transplantation		
Pancreatic islets	Induction of allo-transplantation tolerance, prevention of autoimmune recurrence after syngeneic transplantation, prevention of early xenograft failure	[122,123,134,135]

despite the total elimination of suppressor cells in the $1,25(OH)_2D_3$– treated group by cyclophosphamide, as shown by co-transfer experiments. $1,25(OH)_2D_3$ treatment also did not interfere with the quantitative and qualitative recovery of the major lymphohematopoietic cells after cyclophosphamide injection. Striking again was the absence of insulitis in most animals treated with $1,25(OH)_2D_3$.

The combination of these data (resistance against cyclophosphamide and reduction of insulitis) together with the absence of protection in co-transfer experiments suggested that indeed cyclophosphamide had eliminated all suppressor cells, but that these suppressor cells were not the main protective mechanism in the $1,25(OH)_2D_3$–treated NOD mice.

The basis of protection by $1,25(OH)_2D_3$ seems to be more a reshaping of the immune repertoire with elimination of effector cells, but also the direct β-cell protective effects of $1,25(OH)_2D_3$ might play a major role in disease prevention. The reshaping of the immune system involves more specifically a shift of production of T-cell cytokines from predominantly Th1 (IL-2, IFNγ) in control mice to Th2 (IL4, IL10) in $1,25(OH)_2D_3$ or analog-treated mice [83]. Moreover, this shift appeared to be antigen-specific and most probably due to a direct interference of $1,25(OH)_2D_3$ or its analogs with the antigen presenting dendritic cells. Indeed, $1,25(OH)_2D_3$ induces a reshaping of the dendritic cells towards tolerogenic cells [75,84,85]. We even demonstrated that dendritic cells generated in the presence of $1,25(OH)_2D_3$ or an analog can redirect already committed T-cell clones derived from a type 1 diabetic patient towards nonproliferation [85]. In the NOD mouse, the reshaping of the immune system happens centrally, in the thymus, where treatment with $1,25(OH)_2D_3$ restores the sensitivity of T lymphocytes towards apoptosis inducing signals, thus allowing better elimination of auto-immune effector cells [86].

Studies on diabetes prevention in the NOD mouse model, with spontaneous development of type 1 diabetes, are probably the most relevant for direct application of the findings in the human situation. But the fluctuating incidence of diabetes in the stock mouse colonies and the duration of most intervention studies makes this model not optimal for the screening of large groups of new potentially therapeutic agents. Some researchers have therefore looked for an easier model to study and especially a quicker model for screening new drugs. Such a fast model is the multiple low-dose streptozotocin (MLDSZ) model [87]. Streptozotocin is an antibiotic produced by *Streptomyces achromogenes* that has a specific β-cell toxic effect. A single high dose of streptozotocin (70–250 mg/kg body weight) causes a fast and complete destruction of β-cells in most species.

The administration of multiple subdiabetic doses of streptozotocin causes more subtle β-cell damage, thus triggering a nonspecific inflammatory reaction in the islets that is then followed by insulin deficiency. A major criticism of this model is the fact that the complete scenario of β-cell destruction in this model is unclear and that diabetes is probably the result of nonspecific inflammatory damage of the β-cell together with other islet cells. Therefore, this diabetes model is not a true autoimmune model. However the question remains whether in some humans type 1 diabetes is a true autoimmune disease and not a consequence of nonspecific β-cell destruction by one or other inflammatory conditions (e.g. viral infection).

Inaba *et al.* used the high and the MLDSZ model to test the effect of 1α-hydroxyvitamin D_3, a precursor of $1,25(OH)_2D_3$, on diabetes prevention [88]. When diabetes was chemically induced by a single injection (200 mg/kg body weight) of streptozotocin, no protection against diabetes was seen. When, however, multiple low doses of streptozotocin were administered, 1α-hydroxyvitamin D_3 reduced the diabetes incidence dramatically in a dose-dependent manner: control mice developed diabetes in 100% of the cases, while administration of 0.4 μg/kg of 1α-hydroxyvitamin D_3 reduced the diabetes incidence to 46% ($p < 0.01$). Administration of 0.3 μg also provided protection (diabetes incidence of (61%, $p < 0.025$), but administration of 0.2 μg/kg of 1α-hydroxyvitamin D_3 did not prevent diabetes. Data on toxicity in this study are unfortunately limited to evolution of body weight (unchanged), and no indication of calcemic or bone effects are given. Histologic examination of the pancreas of the experimental mice demonstrated that 1α-hydroxyvitamin D_3 also reduced insulitis in this model.

The BB rat is the other spontaneous animal model for type 1 diabetes. Data on immune pathogenesis of type 1 diabetes in the BB rat are less abundant than in the NOD mouse, and observations are hampered by the severe lymphopenia and T-cell dysfunction occurring in these animals [89–91]. The existence of different pathogenetic mechanisms for autoimmune diabetes in animal models suggests the existence of different pathogenetic scenarios in the human situation. This is also suggested by the apparent contradictory findings sometimes seen in different patient populations, e.g., different genetic characteristics between Japanese and Caucasian diabetic patients, differences in immune characteristics between the patients with very early onset of the disease versus later onset of the disease, and finally the differences observed between patients with isolated type 1 diabetes and patients with multiorgan autoimmune involvement.

In the BB rat, no difference in diabetes incidence between control (33%) and $1,25(OH)_2D_3$–treated rats (24%) was observed when $1,25(OH)_2D_3$ was administered from weaning until 120 days (0.8 µg/kg every other day) [92]. These findings in the BB rat again confirm the basic differences in disease pathogenesis that can be found between the two available animal models for type 1 diabetes and also indicate that caution is warranted when transferring findings from either of these models to the human situation.

2. LATE INTERVENTION — EFFECTS OF $1,25(OH)_2D_3$ ON DIABETES PREVENTION

The NOD mouse is considered a good model for human diabetes and allows for testing not only new therapeutic drugs, but also offers the opportunity to elaborate the optimal and achievable treatment modalities. A relevant question, for instance, is whether long-term $1,25(OH)_2D_3$ treatment is necessary or whether a short-term intervention would suffice for disease prevention and if so, when this short-term treatment should be administered. We, therefore, designed an experiment where NOD mice received $1,25(OH)_2D_3$ in different time windows: one group received $1,25(OH)_2D_3$ for their whole lifetime (from weaning until 200 days of age), another group was treated only during their youth (from weaning until 100 days of age), and a final group was treated only from 100 days of age until 200 days, All mice were followed up until 200 days, and the dose of $1,25(OH)_2D_3$ was 5 µg per kg body weight administered every other day in all groups. A control group vehicle received arachis oil. As expected, insulitis incidence at 200 days was lowered from 93% to 54% ($p < 0.025$) in the long-term treated group while also diabetes incidence was reduced from 86% in this control group to 13% in the $1,25(OH)_2D_3$–treated group ($p < 0.001$). Mice treated only during youth showed a significantly lower insulitis incidence (46%, $p < 0.05$ versus the control group), and diabetes occurred in 30% of these mice ($p < 0.001$). When therapy was initiated at day 100 (when control mice have insulitis in about 75%), insulitis was present in 90% and 80% of these mice developed diabetes by day 200. These values are not significantly different from the control values. When however, not the endpoint of diabetic occurrence but the timing of diabetes onset was compared, the treated animals showed a delayed onset of diabetes compared to the control group ($p < 0.01$). In the group treated from 100 until 200 days of age, effects on serum calcium levels were comparable to the "long-term" treated group; in the group treated from 21 until 100 days of age, the serum calcium level was normal as expected. Bone turnover (reflected in serum osteocalcin levels) in the animals treated with $1,25(OH)_2D_3$ from 21

until 200 days of age was increased as previously demonstrated, while the bone turnover in the two groups with shorter treatment duration was closer to the normal range. In the group receiving the treatment in their youth, these values were still elevated. Bone calcium content determination reflected again the severe impact of $1,25(OH)_2D_3$ treatment in youth since the bone calcium content of these animals even 100 days after stopping the treatment was still significantly decreased compared to the control group ($p < 0.001$). This period seems to be crucial in bone remodeling, and interference with bone modeling in this period leaves traces for the rest of life. On the other hand, when treatment was only initiated at 100 days of age, important bone loss under $1,25(OH)_2D_3$ treatment was seen, although less than with the long-term treatment ($p < 0.01$).

At the moment, clinical trials are underway where $1,25(OH)_2D_3$ is administered to newly diagnosed type 1 diabetic patients, with residual C-peptide levels, and thus residual β cell function [A. Ziegler, P. Pozzilli, personal communication]. Read-out of the trials is preservation of C-peptide levels. Great caution should observed with this kind of trial since the doses administered are at the edge of toxicity, and, as the NOD model suggests, only a delay of disease progression is to be expected.

3. ANALOGS OF $1,25(OH)_2D_3$

A major obstacle to human application of $1,25(OH)_2D_3$ is its important effect on calcium and bone metabolism. New structural analogs of $1,25(OH)_2D_3$ with less effects on calcium metabolism, but more pronounced immunological effects have been developed, especially through side chain modifications [75,93–97]. The actions of this family of molecules on the immune system are exerted via receptors for $1,25(OH)_2D_3$ (VDR) that are present in several immune cells, such as dendritic cells, monocytes/macrophages, activated T lymphocytes and B lymphocytes. *In vivo,* these immune effects result in protection against autoimmunity and prolongation of allograft survival (see chapter 36) [71,72,75,98–104]. Several of the most promising of these analogs coming from different chemical laboratories have been tested in the model of spontaneous type 1 diabetes in the NOD mouse [93]. The mechanism of protection against insulitis and diabetes appears to be similar to that of $1,25(OH)_2D_3$. Effects of the analogs on dendritic cell phenotype, regulator cell induction, and β cell protection have been described [75]. Exposure to analogs of $1,25(OH)_2D_3$ enhances both chemotactic and phagocytotic capacity of monocytes/ macrophages, while their antigen-presenting cell function decreases. Moreover, $1,25(OH)_2D_3$ alters significantly the antigen-presenting cell function of dendritic cells. Treatment with analogs of

1,25(OH)$_2$D$_3$, both during *in vitro* and *in vivo* maturation, inhibits the surface expression of MHC class II and of co-stimulatory molecules, such as CD86, CD80, and CD40 [85].

In the search for the optimal analog a combination of β cell protection, immune modulation and low calcemic effects is sought. Until now, several analogs are promising, but before embarking on long-term interventions in high-risk individuals for type 1 diabetes, long-term safety data will have to be gathered.

4. Combination with other Immune Modulators

In animal models of type 1 diabetes, such as the NOD mouse, disease prevention can be achieved by chronic use of immune suppressants such as cyclospoine CsA ([105]) (Table II). Such an approach cannot be applied to humans because of chronic side effects. Common adverse effects of calcineurin inhibitors like CsA and tacrolimus (FK506) are bowel disturbance, nephrotoxicity, neurotoxicity, as well as serious side effects on pancreatic β cell function, for example, reduced insulin synthesis and β-cell toxicity [106]. Most immunosuppressive agents target T-cells, or some, like mycophenolate mofetil (MMF), both T and B cells. Conversely, no immunomodulatory agent in clinical use specifically targets antigen-presenting cells and in particular dendritic cells, which are known to be involved in T cell activation and tolerance induction.

In NOD mice, diabetes can be prevented by 1,25(OH)$_2$D$_3$ and its nonhypercalcemic analogs when treatment is started before insulitis is present [72,104]. A critical question for the applicability of these analogs in the human situation is whether the analogs of 1,25(OH)$_2$D$_3$ can arrest progression to clinically overt

TABLE II   1,25(OH)$_2$D$_3$ and Its Analogs Combined *In Vitro* and *In Vivo* with Other Immune Suppressants

Drugs		References
	***In vitro* models**	
CsA	Proliferation and IL-2 production of PHA-stimulated PBMC	[108,109,120,136,137]
	Proliferation and cytokine production in MLR	
Rapamycin	Proliferation of PHA-stimulated PBMC	[109,138]
FK506	Proliferation of PHA-stimulated PBMC	[109,120]
	Proliferation and cytokine production in MLR	
MMF	Proliferation of PHA-stimulated PBMC	[109]
Leflunomide	Proliferation of PHA-stimulated PBMC	[109]
Dexamethasone	Proliferation and cytokine production of anti-CD3-stimulated PBMC	[130,140]
	Proliferation, cytokine and chemokine production and T cell activation of dendritic cells	
	Induction of regulatory IL-10 producing T lymphocytes	[141]
	***In vivo* models**	
CsA	Experimental autoimmune encephalomyelitis	[109,142]
	Type 1 diabetes	[108,133]
	Mercuric-chloride induced autoimmunity	[143,144]
	Adjuvant arthritis	[145]
	Thyroiditis	[101]
	Transplantation of syngeneic islets	[110,134,146]
	Transplantation of xenogeneic islets	[135]
	Transplantation of vascularized renal allografts	[147,148]
	Transplantation of liver allografts	[149]
	Bone marrow transplantation	[150]
	Transplantation of aorta allografts	[151]
	Transplantation of heart allografts	[152]
	Transplantation of skin allografts	[153]
Rapamycin	Experimental autoimmune encephalomyelitis	[138]
MMF	Experimental autoimmune encephalomyelitis	[109]
	Transplantation of allogeneic islets	[122]
IFN-β	Transplantation of syngeneic islets	[123]

diabetes if administered when active β cell destruction is already present, which is the situation in prediabetic subjects in whom immune intervention is considered [107]. Casteels *et al.* demonstrated that some of these analogs, when combined with a short induction course of a classical immunosuppressant such as CsA, can arrest the progression of the disease even when administered after autoimmunity has already started [108]. This approach of combining a short induction course with a classical immunosuppressant and a nonhypercalcemic analog of $1,25(OH)_2D_3$ is very promising and might open new perspectives in the prevention of autoimmune diabetes in humans.

Several other combinations of vitamin D analogs and other immune modulators have been tested both *in vitro* and *in vivo*. Using the median effect analysis of Chou and Tallaylay, five top candidates for a combination therapy with $1,25(OH)_2D_3$ could be selected: CsA, FK506, rapamycin, leflunomide, and MMF. These therapies had a respective combinatorial index for 50% suppression of PHA induced human T-cell proliferation of 0.16 ± 0.06; 0.27 ± 0.12; 0.36 ± 0.34; 0.39 ± 0.12 and 0.43 ± 0.30, with index <1 representative for synergism [109]. In the model of experimental autoimmune encephalitis (EAE) in SJL mice the *in vitro* results could be translated to the *in vivo* situation (see Chapter 98). More novel immune modulators, such as type I interferons (IFNα/β), which have a broad range of immunomodulatory properties (mainly inhibition of T-cell IFNγ production by blocking IL-12 secretion of dendritic cells and restriction of T-cell proliferation in part through down-regulation of IL-12 messages or up-regulation of IL-10 levels), also gave interesting results in combination with nonhypercalcemic analogs of $1,25(OH)_2D_3$ in EAE and models of type 1 diabetes [van Etten *et al.* unpublished data and see following paragraph].

B. Secondary Prevention of Type 1 Diabetes in the NOD Mouse by Analogs of $1,25(OH)_2D_3$—Prevention of Recurrence of Autoimmune Diabetes after Islet Transplantation

Type 1 diabetes is characterized not only by an autoimmune destruction of the body's own β-cells, but also by the formation of an autoimmune memory. The latter phenomenon is responsible for the destruction of MHC matched or syngeneic β cells, transplanted under the form of isolated β cells, islets, or whole pancreas [110–116]. This disease recurrence explains why in clinical pancreas and islet transplantation in type 1 diabetic patients, extensive immune suppression is needed: relatively high doses of several immune suppressants

are needed not only to prevent allograft rejection, but also to break the autoimmune memory [117–119].

Some analogs of $1,25(OH)_2D_3$ have been tested for their capacity to prevent disease recurrence after islet transplantation in spontaneously diabetic NOD mice. The most spectacular results were obtained with a combination of KH1060 (20-epi-22-oxa-24,26, 27-trishomo-1,25-$(OH)_2D_3$) (see Chapter 84) and subtherapeutic doses of CsA [120,121]. Administration of high doses of the analog (1 μg/kg/2d) alone was as effective in delaying islet loss as the highest tolerable dose of CsA (15 mg/kg/d), but eventually the disease recurred in all mice. In the group receiving KH1060 (0.5 μg/kg/2d) together with CsA (7.5 mg/kg/d), a synergistic effect between both drugs was seen: 4 of 7 mice maintained a functioning graft for 60 days and more importantly, these animals did not show recurrence for at least 30 days after stopping the treatment. All treatment was administered from the day before transplantation until diabetes recurrence or in the case of normoglycemia until 60 days after transplantation. Insulin content determinations of the graft and native pancreas of the recipient clearly demonstrated that normoglycemia was the result of graft survival and not of recovery of the β-cells of the recipient's own pancreas. Insulin content in pancreases of recurring and nonrecurring mice was comparable and showed noregeneration of the original β-cells (0.0125 ± 0.012 pmol/mg in recurring versus 0.008 ± 0.004 pmol/mg in nonrecurring mice, NS), while the insulin content in the grafts showed a clear difference between recurring and nonrecurring mice (45 ± 27 pmol/graft in recurring versus 1285 ± 106 pmol/graft in nonrecurring mice, $p < 0.00001$).

The highest dose of KH1060, as well as the highest dose of CsA, had clear toxic effects on the general condition of the animals as reflected by the course of the weight. However, by giving KH1060 in a fractionated way (1 μg/kg/2 days instead of 0.5 μg/kg/d) these, cumulative high doses were well-tolerated for long periods of time. The subtherapeutic doses of KH1060 and CsA were nontoxic and had minor effects on serum calcium and osteocalcin levels. The combination of both drugs was also well-tolerated and had similar effects on serum calcium, but resulted in clear effects on osteocalcin levels, indicating also a synergistic effect on bone remodeling. Bone calcium content in all treatment groups was decreased. In the KH1060 groups, this effect was more important and dose-dependent. Unfortunately, as already reflected by the osteocalcin levels, the combined subtherapeutic doses of KH1060 and CsA also act synergistically on bone remodeling. Caution in interpreting these results is warranted since treatment duration in tested animals in the combination group lasted for 60 days, while the animals tested in the other groups only received treatment for shorter periods ($\pm$ 20 days).

Adorini *et al.* have reported a similar synergism between $1,25(OH)_2D_3$ or an analog and mycophenolate mofetil (MMF) [122]. A novel approach can be found in combinations of analogs of $1,25(OH)_2D_3$ with other natural immune modulators such as IFN β. We demonstrated that subtherapeutic doses of rIFN β alone (1×10^5 IU/d) had minor effects on autoimmune diabetes recurrence after islet transplantation (20.8 ± 14.2 days, NS vs. 10.8 ± 2.9 days in controls). However, interestingly, a combination of rIFNβ with TX527 (14-epi-19-nor-20-epi-23-yne-1,25-$(OH)_2D_3$) maintained islet graft function in 100% of mice during treatment (start: the day before islet transplantation and continued until 20 days (rIFNβ) or 30 days (TX527) after transplantation) and resulted in a marked delay of autoimmune diabetes recurrence (61.6 ± 19.6 days, $p < 0.005$ vs. controls) [123]. We also demonstrated that rIFNβ in combination with TX527 results in an inhibition of the Th1 pathway (IL-12, IL-2 and IFNγ), which is known to be associated with the pathogenesis of organ-specific autoimmune diseases. In addition, enhanced expression of Th2 cytokine IL-110 by rIFNβ in combination-therapy with the TX527 analog was observed.

IV. VITAMIN D RECEPTOR POLYMORPHISM AND THE RISK FOR DIABETES

Novel insights on the possible role of vitamin D/$1,25(OH)_2D_3$ in the pathogenesis of type 1 and type 2 diabetes come from the epidemiological data on correlations between VDR polymorphisms and risk of the disease in certain populations. Vitamin D exerts its genomic effects mainly via the nuclear VDR. VDR is expressed in many tissues, including pancreatic islets [30]. The gene encoding the VDR is located in humans on chromosome 12cen-q12 and shows extensive polymorphism including a *FokI* polymorphism in exon II, *BsmI,* and *ApaI* allelic variants in the intron between exons VIII and IX, a *TaqI* restriction fragment length polymorphism in exon IX, and a mononucleotide $[(A)_n]$ repeat polymorphism in the 3′ untranslated region (see Chapter 68). More than 15 reports in different populations show correlations between some of these polymorphisms and type 1 or type 2 diabetes. McDermott *et al.* demonstrated that excessive transmission of the *BsmI* alleles affects South Indian subjects with type 1 diabetes, linking *BsmI* polymorphism of the VDR to an increased risk for type 1 diabetes [124]. This association was confirmed in different populations [125–128]. A study by Ban *et al.* revealed an association between VDR initiation codon polymorphism (*FokI*) and GAD65 positivity in the Japanese

population [129]. The meaning of these correlations between genetic markers of vitamin D metabolism and type 1 diabetes remains unclear. Still, the protective effects of vitamin D on the β cell should not be neglected and might be an explanation of why correlations are observed, not only between VDR polymorphisms and type 1 diabetes but also type 2 diabetes.

Indeed, a study on Bangladesh Asians demonstrated that *ApaI* polymorphisms influence insulin secretion in response to glucose [130]. Also Oh and Barrett-Conner investigated VDR polymorphism and susceptibly for type 2 diabetes in a community-based study of unrelated older adults without known diabetes [131]. Their research suggested that *ApaI* polymorphism may be associated with higher fasting plasma glucose and prevalence of glucose intolerance. More recently, genotyping for *TaqI*, *ApaI*, *BsmI,* and *FokI* revealed that *BsmI* in young males with low physical activity (≤3 h per week) was associated with high levels of fasting glucose [132]. From these data, vitamin D and VDR polymorphisms may have a potential role in the pathogenesis of diabetes. Until now, no clear explanation for these associations has been given. The *FokI* polymorphism could have functional implications, altering ligand-mediated gene expression in β cells or the immune system. An explanation for associations with the polymorphisms located in introns is more difficult to understand.

V. CLINICAL PERSPECTIVES

Clear effects of $1,25(OH)_2D_3$ and its newer analogs have been described on the different major players in the pathogenesis of diabetes mellitus, both type 1 and type 2 diabetes. *In vitro* as well as *in vivo,* a modest stimulation of insulin synthesis and insulin secretion by $1,25(OH)_2D_3$ is observed [31,34,49–52]. This positive effect is not only observed upon repletion of $1,25(OH)_2D_3$ in the vitamin D–deficient state [31,49–51], but can also be observed in the vitamin D–replete state [34,49–52]. Moreover, a direct β cell protection by $1,25(OH)_2D_3$ and its analogs against metabolic and inflammatory stress has been demonstrated [66]. On the other hand, major effects on the immune system involved in the pathogenesis of type 1 diabetes have been described *in vitro* as well as *in vivo* [25,27,28], and prevention of type 1 diabetes and its recurrence after islet transplantation by $1,25(OH)_2D_3$ and its analogs can be achieved (alone or in combination with other immune modulators) [72,89,104,120].

A major problem with using $1,25(OH)_2D_3$ or the currently available analogs in prevention or cure of diabetes is their hypercalcemic and bone remodeling effects when administered in the doses needed for immune or β-cell protective effects. Future applications

of this therapy in human diabetes are conceivable, since through chemical alterations of the $1,25(OH)_2D_3$ molecule even better analogs, with an optimal dissociation between calcemic and immune modulator effects can be synthesized [120].

A place for these analogs in the treatment (prevention or cure) of diabetes can be conceived first of all as β-cell protective and stimulating agents added to the current treatment modalities of type 2 diabetes. Further, these analogs could play a major role in prevention strategies for type 1 diabetes in humans, because of their ideal profile as β-cell protective and especially immune-active drugs. However, before applying these drugs in humans, more information should be gathered not only on their mechanism of action but especially on the safety of these products in long-term use.

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Vitamin D, A Neuroactive Hormone: From Brain Development to Pathological Disorders

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I. INTRODUCTION

The etiology and physiopathology of most neurodegenerative diseases of the central nervous system (CNS) remain poorly understood. In Parkinson's disease (PD), motor impairments result from the selective death of the dopaminergic neurons of the substantia nigra, resulting in a depletion of dopamine in their target area, the striatum. Huntington's disease (HD) is a genetic autosomal dominant disorder with a fatal outcome. It is initially characterized by a loss of γ-amino butyric acid-containing (GABAergic) neurons of the striatum. These neurons constitute a complex population, characterized by the presence of various neuropeptides (neuropeptide Y, substance P) and calcium-binding proteins, such as calbindin-D28k or parvalbumin. GABAergic neurons project their axons towards the substantia nigra, the cortex as well as other structures, and their destruction results in both cognitive and motor impairments. Alzheimer's disease (AD) is a complex pathology characterized by a spatiotemporal extension of two major lesions, the intraneuronal accumulation of paired helical filaments of pathogenic tau proteins and the deposits of amyloid. Both lesions mainly affect the cortex and the hippocampus. Further neurodegenerative phenomena extend in a retrograde manner to other structures, such as to the cholinergic neurons of the septum, diagonal band, and the Meynert's nuclei, which project axons into the hippocampus and cortex.

Glutamate, the major excitatory neurotransmitter in the brain, is known to act as an excitotoxic agent when present in excess, provoking through the N-methyl-D-aspartate (NMDA) receptor a massive entry of calcium, and it generates oxidative or nitrative stresses that ultimately cause neuronal death. Glutamate excitotoxicity may be one mechanism that participates in the neurodegeneration of HD and PD (reviewed in [1,2]). However, reactive oxygen species (ROS) are probably broadly involved in the terminal events leading to cell death. In line with this, post-mortem findings support the involvement of ROS in the progression of PD, HD, and AD (reviewed in [3–5]). Moreover, animal models of PD are based on a selective intoxication of dopaminergic neurons with 6-hydroxydopamine, a compound whose catabolism by monoamine-oxidase generates ROS. The same is true for the 1-methyl-4-phenylpyridinium ion (MPP+), which causes a Parkinson-like syndrome in humans. In AD, the amyloid deposits, which form the senile plaques or more diffuse perivascular deposits, are essentially composed of a 40–42 amino-acid peptide referred to as *amyloid β peptide* (Aβ). Aggregated Aβ peptide has a neurotoxic action, and activates microglial cells, the brain resident macrophages. These cells sustain a protracted pro-inflammatory context within the brain of AD patients, with a liberation of ROS and

cytotoxic lymphokines such as tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1) [3–6]. In multiple sclerosis (MS), demyelination occurs in active plaques, in response to a massive infiltration by macrophages, but also by T lymphocytes, including autoreactive T-cells, which react with antigens present in the myelin sheath, such as myelin basic protein (MBP). In this case, too, oligodendrocyte death is ascribed to the production of ROS in a marked pro-inflammatory context (reviewed in [7]).

The possible involvement of 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$) in MS was proposed on the basis of its action on the immune system, since it was found to have prophylactic effects in experimental autoimmune encephalomyelitis (EAE), a model of MS. Other putative actions of 1,25(OH)$_2D_3$ in the CNS have been ascribed to its classical role in the control of calcium metabolism, for instance *via* the expression of calcium-binding proteins in neurons. However, there is a growing body of evidence which broadens the roles of 1,25(OH)$_2D_3$ in the brain. These include regulatory effects on the production of neurotrophic factors or the tuning of the brain detoxifying machinery. Moreover, neurons, but also astrocytes, oligodendrocytes, and microglial cells are cell targets of this hormone. It seems, therefore, that 1,25(OH)$_2D_3$ is part of a complex cytokine/hormonal network controlling brain homeostasis and repair.

II. VITAMIN D RECEPTOR AND TARGETS IN THE CENTRAL NERVOUS SYSTEM

1,25(OH)$_2D_3$ and other metabolites of vitamin D have been detected in the cerebrospinal fluid, and this hormone has also been shown to cross the blood-brain barrier [8–10]. Furthermore, microglial cells can convert 25-hydroxy vitamin D_3 into 1,25(OH)$_2D_3$ upon activation with interferon-γ (IFNγ) [11]. Detection of 25-hydroxyvitamin D-1α-hydroxylase transcripts in the brain, including in neurons [12,13], supports the concept that the active metabolite of vitamin D can be synthesized in the CNS. In order to identify the hormone's target cells, several studies have been performed using radiolabeled 1,25(OH)$_2D_3$ and have identified high affinity binding sites, most probably the vitamin D receptor (VDR) in the nervous system of various types of vertebrates. In the CNS, these specific binding sites were found in numerous structures [14]. These include motor trigeminal and hypoglossal nuclei, the stria terminalis or reticular nucleus in the thalamus, the amygdala and CA4 part of the hippocampus, and cholinergic nuclei in the telencephalon. These latter mapping data suggest a role for 1,25(OH)$_2D_3$ in

cognitive processes [15], while the presence of binding sites in hypothalamic nuclei were proposed to underlie an involvement of brain 1,25(OH)$_2D_3$ in the neuroendocrine network, for instance through an influence on corticotropin releasing factor-containing neurons and gonadotropin-containing neurons [16]. Similarly, the implication of 1,25(OH)$_2D_3$ in biological rhythms has also been proposed, because the density of binding sites in circumventricular structures is notably higher in species that are subjected to seasonal behaviors [17,18]. In the peripheral nervous system (PNS), binding sites have been detected in the motor neurons of the spinal cord and in the trigeminal ganglia, that is, in cell bodies of sensory neurons [19].

Studies performed with antibodies directed against the VDR have confirmed the widespread presence of VDR-immunoreactivity in many structures of the adult brain [20–23], and data collected with both methods are largely concordant. An interesting finding is that VDR-immunoreactivity has been detected both in the hippocampus and in numerous cortical areas, including the enthorinal cortex [20], which constitutes an essential structure in the cortico-hippocampal connectivity, and is a primary target of neurofibrillary degeneration in AD [24]. In the rat hippocampus, VDR-immunoreactive neurons have been detected along the whole rostro-caudal axis, but with regional variations [22,23]. VDR-expressing neurons were present in the pyramidal layer of the cornu Ammonis (CA1-CA4), the molecular layer of the dentate gyrus and the hilus, a structure in which neurogenesis occurs, even during adult life. Functionality of the VDR was supported by electrophoretic mobility shift assays performed with the osteopontin or osteocalcin vitamin D–responsive elements (VDRE) [22]. It is worth noting that although VDR immunoreactivity was essentially allocated to cells with a neuronal morphology, the presence of a few VDR-immunoreactive astrocytes, as defined by their network of glial fibrillary acidic protein (GFAP), was also detected [22]. Besides these studies reporting the presence of the VDR in the CNS and PNS, immunoreactive material was observed in numerous cell types in the rat or human retina [25,26].

The immunohistochemical detection of the VDR has also been extended to fetal development. Use of gene-targeted mutant mice showed that the VDR gene is turned on as soon as gestational day 11.5 in the developing mouse brain [27]. In the rat embryo, a pronounced expression of VDR-immunoreactivity takes place during the differentiation of many structures and declines to some extent thereafter [28]. This conclusion is supported by the fact that anti-VDR antibody labeled cells were consistently detected in the neuroepithelium and adjacent differentiating fields of developing areas. This temporary expression of VDR was first detected in

the spinal cord and nascent dorsal root ganglia. However, it was found at different time-points in the midbrain and diencephalon, and in the telencephalon, including the cortex and hippocampus. As in the case of the hilar region of the dentate gyrus of the adult rat, expression of the VDR was detected in the ventricular epithelium and the subventricular zones, in which neural stem cells continuously generate migrating neuroblasts which differentiate into olfactory neurons. Finally, expression of the VDR gene has been revealed by *in situ* hybridization, for instance in the pathological human hippocampus [29] or in several areas of the fetal rat brain [28]. This confirms by another method that the VDR is broadly expressed in the nervous system and in the brain, in particular. This expression, however, appears to be tightly regulated by temporal and regional cues. The role of the hormone in the CNS, PNS, or eye remains to be clarified. The fact that VDR immunoreactivity or $1,25(OH)_2D_3$ binding occurs in given neuronal populations does not imply that the hormone directly modulates the neurochemical characteristics or activity of these cells. Rather, $1,25(OH)_2D_3$ might exert a broad action in the brain by influencing basic metabolic functions.

III. VITAMIN D ACTIONS IN THE CENTRAL NERVOUS SYSTEM

A. Calbindins and Other Calcium-Binding Proteins

It has been proposed that $1,25(OH)_2D_3$ could mediate certain effects in the brain by controlling the calcium buffering capacity of neurons through calcium-binding proteins. This was suggested on the basis of the classical role of the hormone in the intestine, kidney, or bone, together with the presence of a canonical VDRE in the promoter of the calbindin-D28k promoter [30]. This possibility was substantiated by the fact that calbindin-D28k transcripts or protein are substantially reduced in certain brain areas in patients deceased from neurodegenerative diseases [29–33]. This is the case of the substantia nigra in PD, the nucleus basalis, and the hippocampus in AD, and the striatum and the hippocampus in HD. One study showed a correlation between reduced calbindin-D28k transcripts and VDR transcripts in the AD hippocampus [29]. However, null mutant mice for the calbindin-D28k gene have an apparently normal CNS, even though they develop a severe ataxia [34]. Furthermore, susceptibility of midbrain dopaminergic neurons to MPP+ is unaffected in these animals [35]. Another fact that indicates that the relationship between calbindin-D28k and $1,25(OH)_2D_3$ is complex in the brain, is that apart from certain

structures, there is no systematic overlap of the presence of calbindin-D28k and that of nuclear and/or cytoplasmic VDR-immunoreactivity [20,21]. A chronic treatment of rats with $1,25(OH)_2D_3$ had no effect on the brain content of calbindin-D28k, protein S100, calmodulin, and parvalbumin, except for the striatum, in which levels of parvalbumin were somewhat increased [36]. In contrast, another study based on shorter treatments detected an accumulation of calbindin-D28k, parvalbumin, and calretinin immunoreactivities in spinal motor neurons [37]. However, investigations with mice lacking the DNA-binding domain of the VDR did not show any alteration in the abundance of calbindin-D28k transcripts in the brain, in comparison to normal animals. In contrast, transcripts of calbindin-D9k were scarcer in mutant animals and treatment with low doses of $1,25(OH)_2D_3$ increased their levels [27]. Paradoxically, this calcium-binding protein is much less represented in the brain than calbindin-D28k, and unlike the latter protein, its expression seems to be subject to nonconventional regulatory mechanisms by the hormone [38]. Hence, these data remain difficult to interpret, especially since these studies used different methodological approaches which preclude their comparison. New clues concerning the role of $1,25(OH)_2D_3$ in the nervous system have been obtained by *in vitro* approaches, from studies performed on primary cultures of brain-derived cells.

B. *In Vitro* Investigations Provide New Clues about the *In Vivo* Actions of $1,25(OH)_2D_3$

Studies performed *in vitro* have shown that rat primary astrocytes and oligodendrocytes contain VDR transcripts, whose abundance rapidly increased in the presence of $1,25(OH)_2D_3$ [39]. Astrocytes also express transcripts of the 25 (OH) vitamin D_3 24-hydroxylase, which do also accumulate in the presence of the hormone [40]. It was concluded that astrocytes have the potential to respond to $1,25(OH)_2D_3$, and that the hormone, which can be synthesized locally [11], also controls to some extent its own catabolism, as in other cells. Likewise, VDR transcripts have been detected in cultures of oligodendrocytes and Schwann cells [41,42], the myelinating cells of the central and peripheral axons, respectively. VDR immunoreactivity has also been identified in cultured hippocampal neurons and in the dopaminergic neurons of the substantia nigra [43,44].

1. EFFECTS ON NEUROTROPHIC FACTORS

A striking response of primary astrocytes, oligodendrocytes, Schwann cells, and CNS neurons to the presence of $1,25(OH)_2D_3$ is a marked increase in the production of the neurotrophic factor nerve growth

factor (NGF). The hormone enhanced both the levels of NGF transcripts and secreted protein [39,41,42,45]. Existence of a promoter in the NGF gene that is activated by the hormone strongly supports the fact that the up-regulation of NGF synthesis in astrocytes, oligodendrocytes, or neurons is primarily of transcriptional origin [46]. Further studies showed that in astrocyte cultures, $1,25(OH)_2D_3$ also enhanced the abundance of neurotrophin 3 (NT-3) transcripts, a neurotrophic protein structurally related to NGF, but appeared to decrease those of brain-derived neurotrophic factor (BDNF), another member of the neurotrophin family [47]. The action of $1,25(OH)_2D_3$ on NGF synthesis has been reproduced in a large variety of transformed cell lines, such as fibroblasts [48,49], osteosarcoma [50], and tumors of neural origin, including C6 gliomas and neuroblastomas [39,51]. The action of the hormone on the production of NGF has also been reproduced *in vivo*. Chronic systemic treatment increased both NGF transcripts and protein in two structures, which naturally produce this factor, the cortex and the hippocampus [52]. Conversely, newborn rats that developed in vitamin D–deficient dams had lower levels of NGF in their brain [53]. These observations are relevant to the clinic, since neuroprotection of the cholinergic, NGF-responsive neurons of the basal forebrain could prevent or delay the progression of mild cognitive impairments in normal aging or AD onset. These observations also provide an explanation for the fact that intraperitoneal or intraventricular treatments with $1,25(OH)_2D_3$ enhanced the specific activity of choline acetyltransferase, as forebrain cholinergic neurons are responsive to NGF [54].

Besides its effects on neurotrophins, $1,25(OH)_2D_3$ has been reported to enhance the expression of another potent trophic factor active on several CNS neurons, the glial cell line-derived neurotrophic factor (GDNF). This was initially reported to take place in rat C6 glioma cells [55], but was not supported by data collected in primary cultures of glial cells. These latter studies, however, detected a potent action of thyroid hormone [56]. Nevertheless, lower levels of GDNF were observed in the brain of vitamin D–depleted newborn rats, as for NGF [53]. Conversely, systemic administration of $1,25(OH)_2D_3$ for eight days increased the GDNF content in the adult rat cortex, while reducing cortical infarction following ischemia [57]. A similar treatment also attenuated the toxicity of 6-hydroxydopamine on a classical target of GDNF, the mesencephalic dopaminergic neurons of the substantia nigra [58].

2. REGULATION OF THE LOW-AFFINITY NEUROTROPHIN RECEPTOR

These *in vivo* data indicate that $1,25(OH)_2D_3$ has the potential to participate in the modulation of neuronal plasticity and survival through an action on the supply of trophic factors, such as NGF and GDNF. However, other mechanisms could operate in parallel. As a mirror image of the effect of $1,25(OH)_2D_3$ on NGF synthesis, the hormone is also involved in the regulation of its low-affinity receptor, referred to as p75[NTR]. This receptor is not restricted to a single ligand, as it binds various neurotrophins. However, it acts primarily as a co-receptor of the different high affinity neurotrophin receptors, the transmembrane tyrosine-kinases TrkA, B, and C (specific binding sites for NGF, BDNF, or NT3, and NT4/5, respectively). The major trophic actions of neurotrophins, such as cell survival, are mediated by Trk receptors. However, these effects are enhanced when the p75[NTR] is present. The affinity of NGF binding is increased when p75[NTR] and TrkA are co-expressed [59]. Furthermore, both receptors reciprocally modulate their transducing machineries in a complex cross-talk [60]. p75[NTR] also participates in the retrograde axonal transport of neurotrophins [61,62]. In contrast, p75[NTR] by itself triggers an opposing signal since it promotes cell apoptosis, instead of cell survival *via* the Trk receptor [63–65]. Up-regulation by $1,25(OH)_2D_3$ of p75[NTR] transcripts was first reported in C6 gliomas and subsequently in cultured oligodendrocytes [42, 66]. A VDRE was also identified in the promoter of the p75[NTR] [66]. Treatment of adult rats with high doses of $1,25(OH)_2D_3$ decreased the levels of p75[NTR] transcripts in the adult spinal cord, thus suggesting a hormone-mediated reduction in the pro-apoptotic potential of this receptor [66].

Conversely, p75[NTR]-immunoreactivity and transcripts were greatly decreased in several brain areas of rat pups born to vitamin D–depleted mothers. This loss of expression could affect the cell death processes that normally occur during brain development [53]. The frequency of proliferating cells in the dentate gyrus, hypothalamus, and basal ganglia was higher in vitamin D–depleted rats. This effect suggests that $1,25(OH)_2D_3$, which has a marked differentiating and antiproliferative action on a large variety of cells, is actively involved in the control of cell number in the brain. Hence, reduced apoptosis associated with an enhanced growth potential would account for the severe alteration in brain shape in vitamin D–depleted pups [53]. It was argued that these observations are relevant with respect to psychiatric disorders, such as schizophrenia, which could have an increased risk factor upon vitamin D deficiency during pregnancy [67,68].

3. CALCIUM CHANNELS

The studies that have focused on the intracellular control of calcium concentration have detected evidence for a modulatory action of $1,25(OH)_2D_3$ on L-type voltage-sensitive calcium channels in cultures of

hippocampal neurons. Prolonged treatment of neurons with low concentrations of the hormone depressed the channel density and mRNA content of the pore forming subunits α1c and α1d. This accounts for the protective effect of the hormone against toxic stimuli such as medium exchange or treatments with NMDA or glutamate [43]. Voltage-sensitive L-type channels are of considerable physiological importance in the function of the hippocampus, including long-term potentiation or gene expression. The regulatory action of $1,25(OH)_2D_3$ provides a molecular explanation for the fact that protracted treatment of rats with the hormone prevents age-related loss of hippocampal neurons [69], and this neuroprotection raises intriguing questions regarding the prevention of the exacerbated degenerative process which operates in the hippocampus of AD patients.

4. $1,25(OH)_2D_3$ AND BRAIN DETOXIFICATION

Another protective action of $1,25(OH)_2D_3$ relies on its regulation of the detoxifying machinery. The hormone was reported to enhance the specific activity of γ-glutamyl transpeptidase in the brain [70]. This enzyme cleaves extracellular glutathione, thus favoring the uptake of catabolic products for the intracellular synthesis of this same compound. In cultures of primary astrocytes, $1,25(OH)_2D_3$ increases the cellular content in glutathione and enhances γ-glutamyl transferase specific activity under severe proinflammatory conditions, without any effect on other enzymes, such as superoxide dismutase or glutathione peroxidase [71]. Type II nitric oxide synthase (NOS II), an inducible enzyme which generates high quantities of NO and which is tightly associated with the physiopathology of MS, is another target of $1,25(OH)_2D_3$. The hormone down-regulates NOS II expression in different models of brain inflammation, such as EAE and lipopolysaccharide (LPS) injection [72,73]. In this latter system, NOS II is first expressed by macrophage/microglia and then by astrocytes. The inhibitory effect of $1,25(OH)_2D_3$ reduces both the abundance of NOS II transcripts and protein. It has been suggested that the regulatory effect is transcriptional and results from the presence of a VDRE in the promoter of the NOS II gene [74]. Since both activated macrophages and microglial cells can synthesize $1,25(OH)_2D_3$ from its precursor, it seems likely that the hormone acts as an endogenous regulator of NOS II in the brain. However, $1,25(OH)_2D_3$ could exert other protective actions against insults generated by ROS. Treatment of mesencephalic neurons with the hormone has been shown to enhance their resistance to 6-hydroxydopamine, MPP^+, glutamate, a calcium ionophore, and H_2O_2 [44,58]. This effect can be achieved following a hormonal pretreatment of at least 24 h and requires protein synthesis. It is not thought to

result from a variation in glutathione levels, but could depend on other thiol-based scavengers, such as thioredoxin or metallothionein [44].

IV. $1,25(OH)_2D_3$ AND BRAIN TUMORS

$1,25(OH)_2D_3$ and its analogs are known to inhibit growth and to induce the differentiation of several cancer cells [75] (see section IX). In CNS tumors, VDR expression has been documented in human meningiomas and gliomas [76–78], which are both nonneuronal tumors. In gliomas, VDR expression was found to correlate with the grade of the tumor with the highest level of expression found in untractable glioblastomas [79]. Several studies have reported the responsiveness of gliomas to $1,25(OH)_2D_3$ or to some of its analogs *in vitro*. Thus, rat or human glioblastoma cell lines respond to the addition of $1,25(OH)_2D_3$, alone or in combination with retinoic acid, by the induction of cell death/differentiation programs [78,80–82]. Hence, it has been suggested that the capacity of activated microglial cells to produce $1,25(OH)_2D_3$ [11] could be part of the mechanisms limiting the extension of some gliomas *in vivo*. A striking observation, which supports a close relationship between $1,25(OH)_2D_3$ and the growth of malignant gliomas, is that amplification of splice variants of the 25-hydroxyvitamin D-1α-hydroxylase gene is a frequent aberration in these tumors [83,84]. However, the responsiveness to the hormone is complex. The finding that the neurotransmitter noradrenaline inhibits $1,25(OH)_2D_3$-induced glioma cell death suggests that if such a cytotoxic mechanism exists *in vivo*, it might be inoperative in the close vicinity of noradrenergic neurons [85]. Furthermore, variants of C6 gliomas, which fail to express the VDR, escape the cell death program triggered by the hormone [86].

Studies of the *in vivo* effects of $1,25(OH)_2D_3$ on gliomas are scarce. However, it is noteworthy that long-lasting complete regression of glioblastomas has been obtained in humans under long-term 1-alpha-hydroxycholecalciferol therapy [87]. Regarding the effects of $1,25(OH)_2D_3$ on tumors of neuronal origin (i.e., neuroblastomas), several studies have reported differentiation or antiproliferative effects of the hormone on neuroblastoma cell lines [88,89]. A synergistic effect with retinoids has also been observed, suggesting the potential use of $1,25(OH)_2D_3$ and retinoid analogs in combination in the clinic [90]. Taken together these results suggest that meningiomas, gliomas, and neuroblastomas are potential targets of $1,25(OH)_2D_3$. It can be expected that in the near future, gene expression profiles of brain tumors will tell us whether VDR expression, alone or with other members of the steroid-thyroid

hormone receptor superfamily, is a valuable diagnostic or prognostic parameter for the use of $1,25(OH)_2D_3$ as a curative compound in these malignant diseases. However, recent data indicating that the hormone plays an active role in the control of cell proliferation during normal brain development [53], raise the possibility that it also contributes to the natural brain defense against carcinogenesis.

V. $1,25(OH)_2D_3$, A MEDIATOR OF NEURO-IMMUNE INTERACTIONS

MS is a slowly progressive, disabling disease of the CNS characterized by disseminating patches of demyelination in the brain and spinal cord. While the etiology of MS is unknown, it is regarded as a complex multi-causal disease comprising genetic factors, dysfunction of the immune system (auto-immunity), and environmental factors. The concept that vitamin D represents an important factor in MS pathophysiology is quite old [91], but has been re-emphasized in more recent days [92,93]. Both the natural immunomodulatory functions of $1,25(OH)_2D_3$ and its beneficial role in the T-cell mediated MS paradigm, EAE, support the protective involvement of $1,25(OH)_2D_3$ in MS (see Chapters 36 and 98).

The hormone was initially described as a preventive factor for EAE when administered at the time of immunization, or to inhibit its passive cellular transfer [94,95]. Further studies showed later that $1,25(OH)_2D_3$ reversibly blocks the progression of EAE when administered after the onset of clinical signs in both rats [96] and mice [97]. Alternatively, vitamin D deprivation aggravated the clinical symptoms of EAE [74,97]. Furthermore, the VDR appeared to be essential for the mediation of the hormonal effects on EAE symptoms [98]. It is now well documented that EAE is mediated by CD4^{+} Th1 cells that secrete proinflammatory cytokines, and the recovery as well as the remission phase is related to CD4^{+} T-cells that secrete transforming growth factor-β1 (TGF-β1) [99].

One mechanism explaining the effect of $1,25(OH)_2D_3$ during EAE includes its regulatory actions in peripheral lymphoid organs. $1,25(OH)_2D_3$ inhibits IL-12 production, which normally promotes Th1 polarization [100,101], and it enhances the lymph node content of the anti-encephalitogenic cytokines TGF-β1 and IL-4 [102]. It also favors the differentiation of monocytes into macrophages [103,104], modulates the expression of co-stimulatory signaling receptors [105], and prevents the maturation of dendritic cells, thus leading to impaired T-cell activation [106–109]. $1,25(OH)_2D_3$ also exerts direct effects on T-cells by inhibiting IL-2

and IFN-γ production [110–113]. Another effect of $1,25(OH)_2D_3$ curative treatments on EAE, is the decrease in expression of the CD4 and MHC class II antigens by brain-infiltrating leukocytes [96].

In parallel to systemic actions, other effects of $1,25(OH)_2D_3$ could directly take place in the CNS. The increase in the anti-encephalitogenic cytokine TGF-β1 in secondary lymphoid organs [102] is probably not associated with a corresponding response in the CNS, since no changes in TGF-β1 secretion were seen in murine microglial cells *in vitro* after exposure to $1,25(OH)_2D_3$ [114], while TGF-β1 transcripts were unaffected in the CNS of EAE rat treated with the hormone [74]. In contrast, down-regulation of NOS II mRNA and protein by $1,25(OH)_2D_3$ [72–74] is likely to be a direct response, since it sequentially affects microglial cells and astrocytes, which are both VDR-responsive cells [39,114], and also takes place following a direct intrahippocampal injection of LPS [72]. The early down-regulation of MHC class II expression on microglial cells observed during EAE may also result from specific CNS actions of the hormone [74,96]. This effect could either be direct, via the VDR, or indirect, involving, for instance, an up-regulation of NGF synthesis by glial cells or neurons [39,42,45,52], which would in turn inhibit MHC class II molecule induction *via* a p75[NTR] receptor-dependent mechanism [115]. Moreover, $1,25(OH)_2D_3$ down-regulates macrophage-colony stimulating factor (M-CSF) and TNF-α production by cultured astrocytes submitted to an inflammatory stress [116]. This observation also supports a pivotal role for the hormone within the CNS itself during EAE, notably on the degree of activation of microglial cells and their ability to serve as antigen-presenting cells. In parallel with microglial cells and astrocytes, the involvement of oligodendrocytes and neurons as targets of $1,25(OH)_2D_3$ during EAE remains unknown.

VI. CONCLUSION

The pleiotropic actions of $1,25(OH)_2D_3$ are summarized in Fig. 1. The hormone clearly behaves as a neuroactive compound largely implicated in the control of brain homeostasis. Because of the potential toxic effects of high doses of $1,25(OH)_2D_3$, several vitamin D analogs (see Section VIII) have been tested in EAE and have been able to reduce the gravity of EAE in a similar fashion to $1,25(OH)_2D_3$, involving notably an inhibition of Th1 development [74,117,118]. Further clinical trials will determine whether these and other analogs exert beneficial effects in resolving MS. Likewise, the neuroprotective effects of the hormone, achieved by its action on the levels of NGF, GDNF,
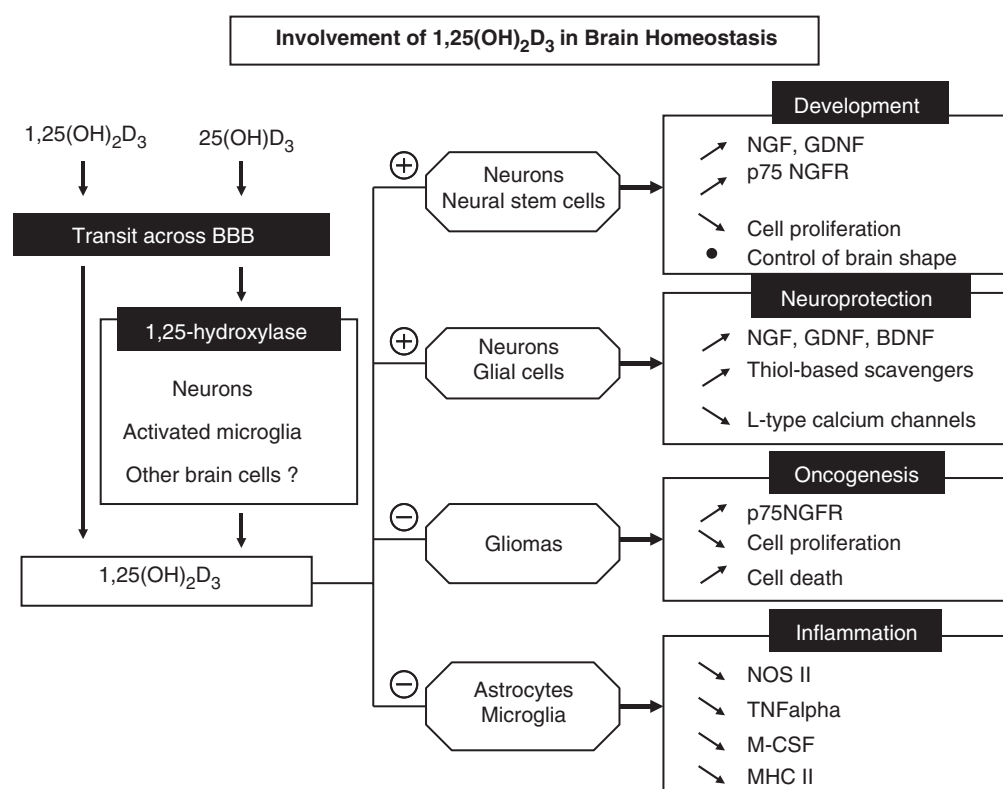
Involvement of 1,25(OH)$_2$D$_3$ in Brain Homeostasis

FIGURE 1 Whilst 1,25(OH)$_2$D$_3$ and its precursor 25(OH)D$_3$ can transit across the blood-brain barrier, an intracerebral synthesis of the active hormone appears possible, since neurons or activated microgial cells express 25-hydroxyvitamin D-1α-hydroxylase (1,25-hydroxylase). The hormone receptor, the VDR, is found both in neurons and glial cells, and the figure summarizes the broad spectrum of responses triggered by the hormone in these cells. Taken together, these responses suggest that 1,25(OH)$_2$D$_3$ contributes to maintaining brain homeostatis, and is also one of the elements conferring special immune status to the CNS.

and the low-affinity neurotrophin receptor support the potential of vitamin D analogs in the treatment of neurodegenerative disorders such as PD and AD. Such analogs could contribute to improving the survival of basal forebrain cholinergic neurons [119] and mesencephalic dopaminergic neurons [120], respectively. Conversely, the contrasting action of 1,25(OH)$_2$D$_3$ on NOS II synthesis and on the detoxifying machinery of brain cells could be beneficial in neurodegenerative disorders or following ischemic injury [1–5,121–124]. An important nutritional issue, however, is to avoid vitamin D deficiency [125–128] in homebound patients or aging persons, as discussed elsewhere in this volume (Chapter 66).

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Psoriasis and Other Skin Diseases

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I. INTRODUCTION

Earlier in the last century, vitamin D$_3$ was used in dermatology in huge pharmacological doses for the treatment of scleroderma, psoriasis, lupus vulgaris, and atopic dermatitis. A rationale for the use of vitamin D in psoriasis was the clinical observation that this disease, in general, markedly improves in the summer. In 1936, J. Krafka wrote in *The Journal of Laboratory and Clinical Medicine* under the headline "*A simple treatment for psoriasis*": "*A commonly observed fact in the South concerning this disease (psoriasis) is that it generally clears up in the summer sun. This led the author to the hypothesis that it might be cured with viosterol…. A patient with a case of ten years standing, continuous duration was put on viosterol (an oil containing vitamin D$_2$) …. Within sixty days from the beginning of the test, the skin of the patient was entirely clear*" [1]. In 1950, H.W. Spier reported results of a study with 94 psoriasis patients performed in 1948–49 [2]. First, patients received orally 30 mg vitamin D$_2$/week for 2–4 weeks, and thereafter 20 mg vitamin D$_2$/week until a total dose of appr. 300 mg vitamin D$_2$ was reached. In this study, patients were treated for 3–4 months; 20% of patients showed a good response, 25% a satisfactory response, 25% a moderate response, and 30% a nonsatisfactory response.

But these first attempts to use vitamin D treatment in dermatology were abandoned because of severe vitamin D intoxication resulting in hypercalcemia, hypercalciuria, and kidney stones occurring in patients that received huge pharmacological doses of vitamin D (up to 1000-fold the regular daily need of vitamin D). Also, they were stopped because other new treatments were introduced for the therapy of these diseases.

II. PATHOGENESIS OF PSORIASIS

A. Psoriasis: Pathogenesis, Immunology, and Histology of Skin Lesions

Psoriasis vulgaris is a chronic dermatosis of unknown etiology characterized by hyperproliferation and inflammation of the skin. The most common form of the disease is plaque psoriasis, in which skin develops scaly, raised red lesions. The severity of chronic plaque psoriasis ranges from mild, when the disease has only a mild impact on quality of life, to severe, when patients' lives are significantly affected [3]. In severe cases, most of the body surface, including the scalp and nails, may be involved. The peak age of onset for this psychologically debilitating and disfiguring disease is the second decade, but psoriasis may first appear at any age from infancy to the aged [4]. It is considered a multifactorial disease and has a prevalence of about 1–2% in the United States and Europe. Population, family, and twin studies clearly demonstrate that there is a strong but very complex genetic component leading to the development of psoriatic skin lesions [5]. Most likely, multiple genes are involved in the pathogenesis of psoriasis. Molecular biology techniques have been developed that allow studies to analyze psoriasis susceptibility genes, but to date, no specific genetic marker of the disease has been identified. Psoriasis has long been known to be associated with certain HLA antigens, particularly HLA-Cw6, although there is no evidence that a psoriasis susceptibility gene exists at this locus [6]. It is unknown what cell types in human skin are primarily affected by the disease.

Recent studies support the hypothesis that epidermal hyperproliferation in psoriasis may be mediated by cells of the immune system, most likely T lymphocytes [7,8].

The vast majority of T-cells in psoriatic lesions are situated in the perivascular area in the dermis; many are also found in the epidermis. Activated CD4+ and/or CD8+ T-cells in psoriatic lesions express HLA-DR, the interleukin-2 receptor (CD 25), bear the CLA+ memory-effector CD45RO+ phenotype, and secrete specific immune mediators and cytokines, such as IL-2 and interferon-γ [8–11]. Thus, psoriasis represents mainly a Th1 profile disease (characterized by T-lymphocyte secretion of IL-2, IL-12, and interferon-γ) [12]. In contrast, atopic dermatitis represents a Th2 profile disease that is characterized by T-cell secretion of IL-4, IL-5, and IL-10 [13]. The activation signal for the development of psoriatic lesions is still unknown, although there is increasing evidence that superantigens such as the N-terminal component of bacterial M-proteins may be of importance for the initiation of T-cell proliferation in psoriasis [8,14]. It has also been hypothesized that psoriasis patients develop an effector-immune response to skin (auto)antigens, which have yet to be specifically identified. According to this model, the immunologic process underlying psoriasis begins with a sensitization-type phase during which the skin dendritic cells migrate to regional lymph nodes where they present these skin antigens to naïve T-cells. This sensitization phase occurs prior to the development of skin lesions [8,15]. When sensitization is obtained, the psoriasis skin lesion may develop as a result of the migration of T-cells in the skin where they are activated by antigen presenting cells, including Langerhans' cells presenting self-skin antigens [8,15].

The histologic appearance depends on the age of the psoriatic lesion and the site of the biopsy. In general, epidermal hyperplasia is present, in which the granular layer may be lost and the stratum corneum shows parakeratosis. Typical lesions show histological elongation of the dermal papillae, with a relatively thin epidermis at the top of the papillae. The epidermis may show in suprapapillar compartments intercellular edema and infiltration with T-lymphocytes and neutrophiles, which can extend into spongiform pustules of Kogoj or Munro microabscesses [16].

III. THE VITAMIN D SYSTEM IN NORMAL AND PSORIATIC SKIN

Vitamin D is made in the skin as a result of exposure to solar or artificial ultraviolet B radiation as discussed in Chapter 3 [17,18]. It is now known that the skin itself is a target tissue for the secosteroid hormone 1α,25-dihydroxyvitamin D (1,25(OH)$_2$D, calcitriol), the biologically active vitamin D metabolite [19,20]. 1,25(OH)$_2$D$_3$ exerts genomic and nongenomic effects.

Nongenomic effects of 1,25(OH)$_2$D$_3$ and analogs are related to effects on intracellular calcium [21,22]. In keratinocytes and other cell types, 1,25(OH)$_2$D$_3$ rapidly increases free cytosolic calcium levels [21,22] (Chapter 23). Genomic effects of 1,25(OH)$_2$D$_3$ are mediated via binding to a nuclear receptor protein (vitamin D receptor; VDR) that is present in target tissues and binds 1,25(OH)$_2$D$_3$ with high affinity (K$_D$ 10^{-9}–10^{-10} M) and low capacity [23,24] (Chapters 11,13). The human VDR has been cloned [25] and sequence analysis demonstrated that this protein belongs to the superfamily of trans-acting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors and the retinoic acid receptors [25] (Chapter 11). Interaction of 1,25(OH)$_2$D$_3$ with VDR results in the phosphorylation of the receptor complex that in turn activates the transcription of 1,25(OH)$_2$D–sensitive target genes, especially genes involved in cellular differentiation and proliferation. VDR requires auxillary factors for sufficient DNA-binding [26]. These auxillary proteins were identified as
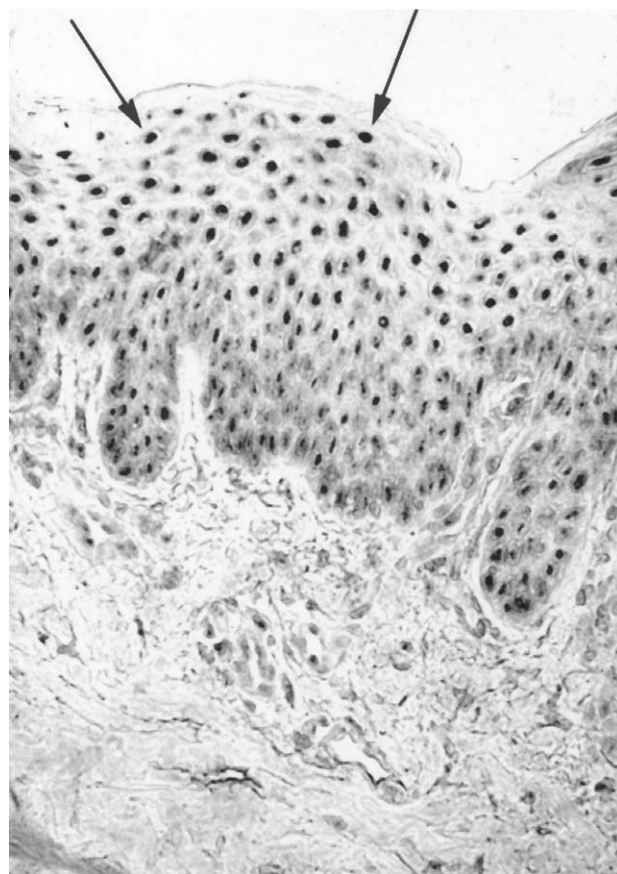

FIGURE 1 Immunohistochemical demonstration of VDR in human skin. Notice strong nuclear VDR immunoreactivity in cells of all layers of the viable epidermis (arrows). Labeled avidin-biotin technique using antibody 9A7γ directed against VDR. Original magnification ×400.

the retinoid-X receptors (RXR-α,-β,-γ) that were shown to heterodimerize with VDR, and facilitate DNA-binding to the respective vitamin D response elements (VDRE) in the promoter region of target genes [26,27]. In the skin, both VDR (Fig. 1) and RXR-α are expressed in keratinocytes, fibroblasts, Langerhans cells, sebaceous gland cells, endothelial cells, and most cell types related to the skin immune system [28,29].

In vitro studies revealed that $1,25(OH)_2D_3$ is extremely effective in inducing the terminal differentiation and in inhibiting the proliferation of cultured human keratinocytes in a dose-dependent manner [30–32] (Chapter 35). Additionally, $1,25(OH)_2D_3$ acts on many cell types involved in immunologic reactions, including activated T and B lymphocytes, macrophages, and Langerhans cells [33,34] (Chapter 36). Data about the effects of $1,25(OH)_2D_3$ on the melanin pigmentation system are still conflicting, but most studies do not support the possibility that $1,25(OH)_2D_3$ regulates melanogenesis in human skin [35].

IV. PHYSIOLOGICAL AND PHARMACOLOGICAL ACTIONS OF VITAMIN D ANALOGS IN NORMAL AND PSORIATIC SKIN

A. Biological Effects of Vitamin D and Analogs in Psoriasis

The mechanisms underlying the therapeutic effectiveness of vitamin D and its analogs in psoriasis are still not completely understood (Fig. 2). The analogs are described in Section VIII of this book. Calcipotriol, which is used for the topical treatment of psoriasis, is discussed in Chapter 84. Results from immunohistochemical and molecular biology studies indicate that the antiproliferative effects of topical $1,25(OH)_2D_3$ on epidermal keratinocytes are more pronounced as compared to effects on dermal inflammation (Fig. 3). Modulation of various markers of epidermal proliferation (proliferating cell nuclear antigen, Ki-67 antigen), and differentiation (involucrin, transglutaminase K, filaggrin, cytokeratins 10,16) in lesional psoriatic skin after topical application of vitamin D analogs was shown *in situ* [36] (Fig. 4). Interestingly, effects of topical treatment with vitamin D analogs on dermal inflammation are less pronounced (CD-antigens, cytokines, HLA-DR, etc.) as compared to effects on epidermal proliferation or differentiation. One reason for this observation may be that the bioavailability of this potent hormone in the dermal compartment may be markedly reduced as compared to the epidermal compartment [36].
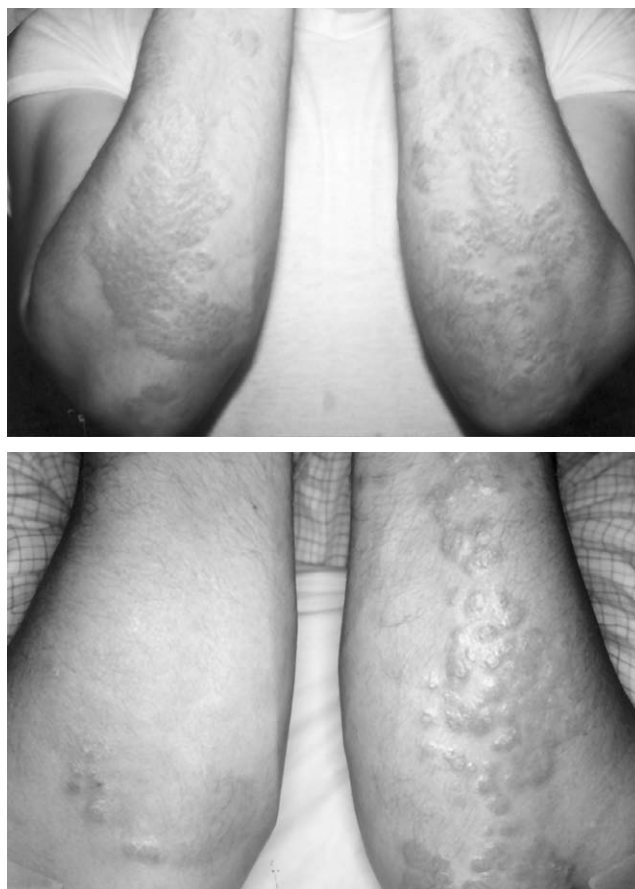

FIGURE 2 (**Top**) shows the arms of a patient with a long history of plaque psoriasis before treatment. (**Bottom**) shows the same patient, who applied only petroleum jelly to the forearm on the right and petroleum jelly containing $1,25(OH)_2D_3$ (15 μg/g) to the forearm on the left.

Molecular biology studies have demonstrated that clinical improvement in psoriatic lesions treated with $1,25(OH)_2D_3$ correlates with an elevation of VDR mRNA [37]. It is well known that some patients suffering from psoriasis either are resistant to or develop resistance to $1,25(OH)_2D_3$ treatment. It was demonstrated that responders can be distinguished from the nonresponders on the molecular level since nonresponders show no elevation of VDR mRNA in skin lesions during treatment. These data suggest that the ability of $1,25(OH)_2D_3$ to regulate keratinocyte growth is closely linked to the regulation of VDR expression. The target genes of topical $1,25(OH)_2D_3$ that are responsible for its therapeutic efficacy in psoriasis are still unknown. Major candidates for $1,25(OH)_2D_3$ target genes that are responsible for the $1,25(OH)_2D_3$–induced terminal differentiation in keratinocytes are distinct cell cycle associated proteins (i.e., INK4 family), including p21/WAF-1 [38].
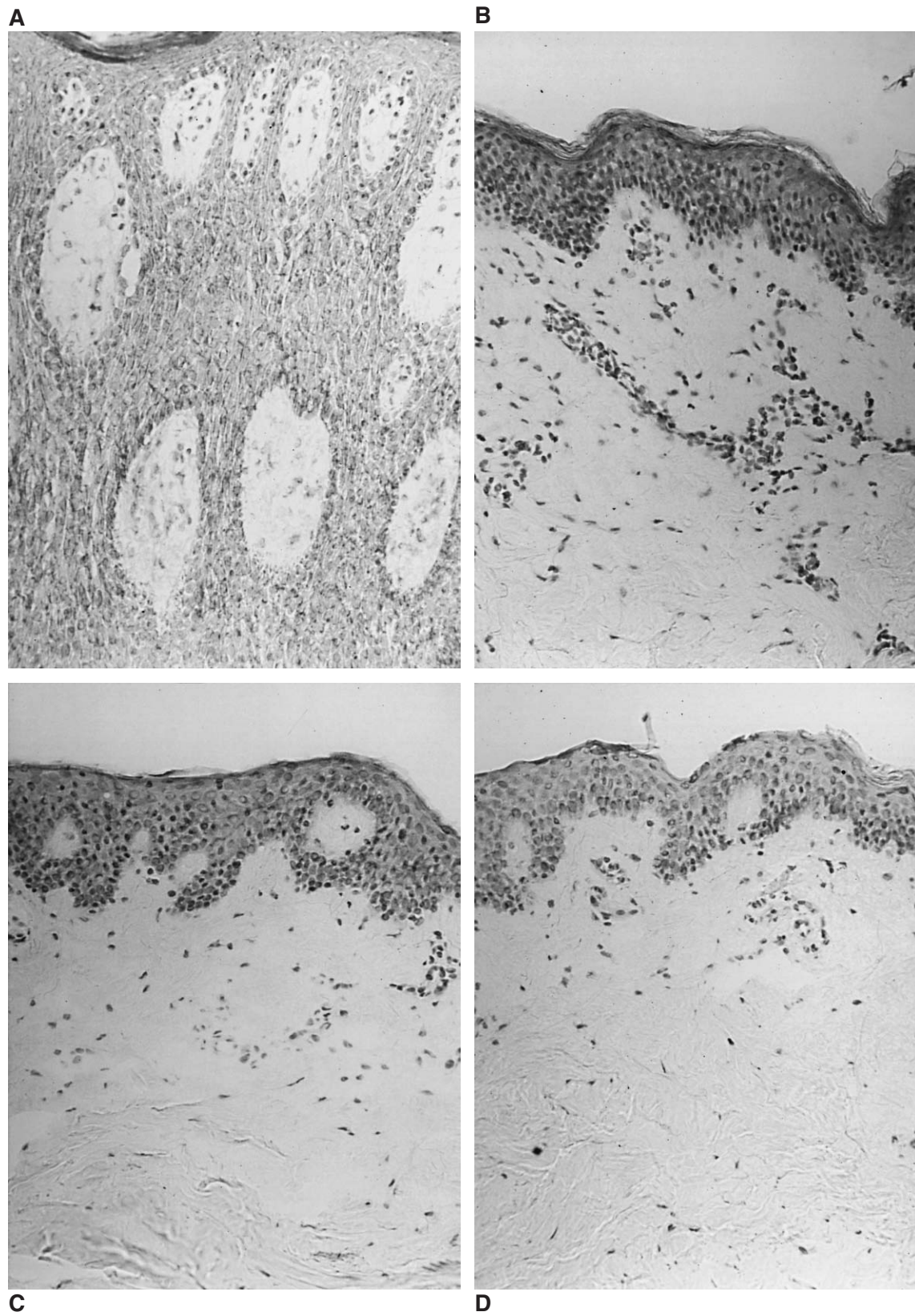
FIGURE 3   Histological demonstration of morphological changes in lesional psoriatic skin after six weeks of topical treatment. **A.** lesional psoriatic skin before treatment, **B.** calcitriol 15 μg/g; **C.** calcipotriol 50 μg/g; **D.** nonlesional psoriatic skin. Notice strong reduction of epidermal thickness after topical treatment with vitamin D analogs. Hematoxylin-Eosin staining. Original magnification ×200. (See color plate).
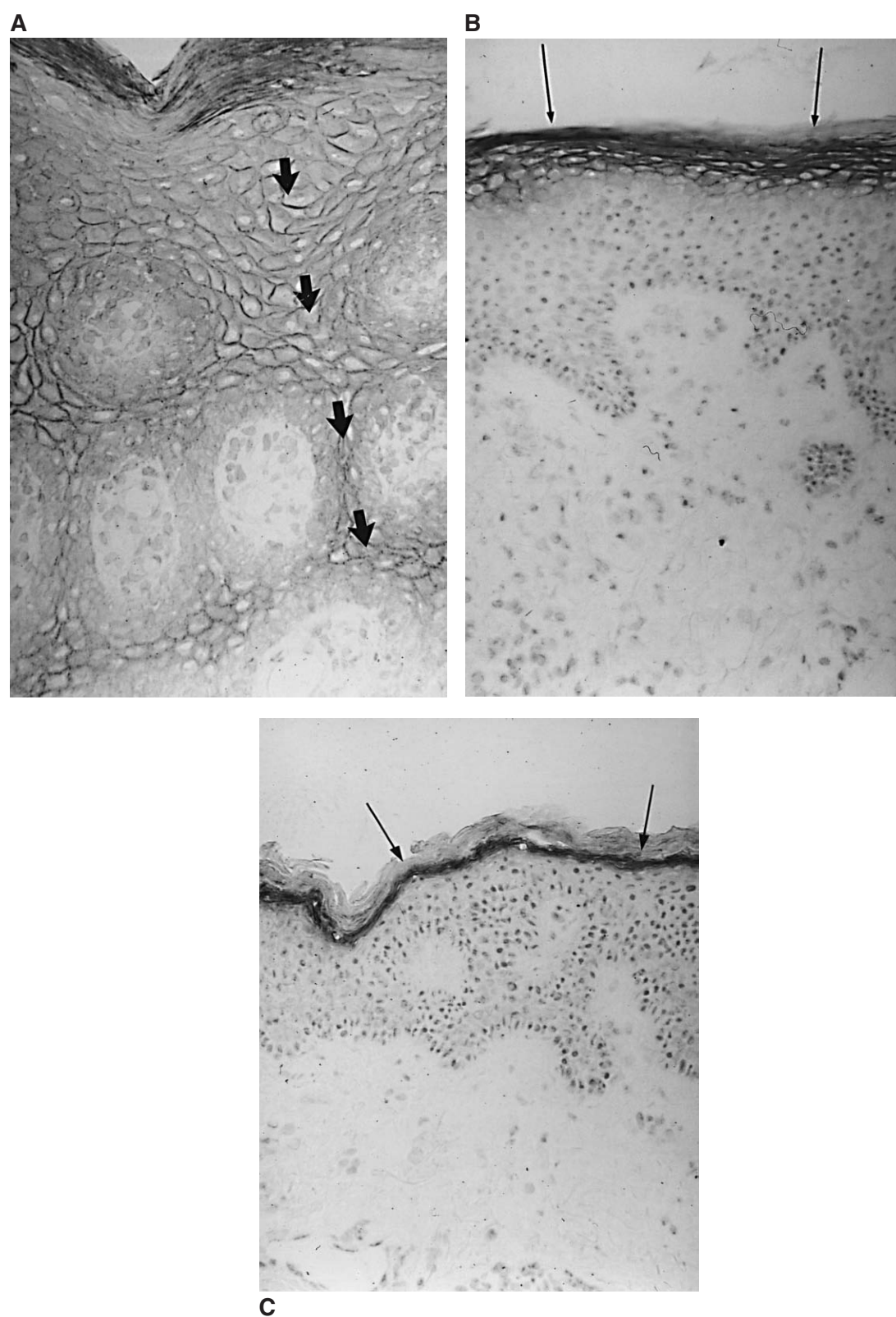
FIGURE 4   Immunohistological detection of transglutaminase K in lesional psoriatic skin. (**A**) before treatment; (**B**) lesional psoriatic skin after six weeks of topical treatment with calcipotriol (50 μg/g); and (**C**) in nonlesional psoriatic skin. Notice strong staining for transglutaminase K in all epidermal cell layers of lesional psoriatic skin before treatment (**A**, arrows). In contrast, after six weeks of topical treatment with calcipotriol staining in lesional psoriatic skin (**B**, arrows) is restricted to the upper layers of the viable epidermis, a staining pattern that is characteristic for nonlesional psoriatic skin (**C**, arrows). Original magnification ×160. (See color plate).

B. VDR Genotypes and Response to Treatment

Data analyzing VDR genotype in psoriasis are somewhat conflicting—some studies report a correlation between individual VDR genotypes and the skin eruptions of psoriasis [37,39]. The VDR genotype are described in detail in Chapter 68. While no differences in VDR genotype between controls and psoriasis patients were reported at the Bsm-1 site, some studies reported a significant difference in terms of the Apa-1 [40] and Fok-1 [41] sites. Additionally, it has been shown that VDR genotypes are not associated with clinical response to calcipotriol, at least in Korean psoriasis patients [42]. Kontula *et al*. [43] and Mee *et al*. [44] studied the Bsm-1 polymorphism and the response to calcipotriol treatment in psoriatic patients and found no association between them. Recently, it has been shown that the frequency of the F-allele (Fok-1 site) was lower in patients nonresponsive to topical treatment with tacalcitol compared with controls (47 vs. 64%, $p = 0.05$). Although the number of patients examined in this study was small, this result is intriguing because the F-allele has been demonstrated to be more responsive to $1,25(OH)_2D_3$ than F-allele *in vitro* (see Chapter 13 and 68). Hutchinson *et al*. [45] reported that the F-allele was significantly less common in malignant melanoma cases than controls, suggesting consistency with *in vitro* data. According to Colin *et al*. [46], the Fok-1 polymorphism was associated with the response to $1,25(OH)_2D_3$, and under conditions of vitamin D insufficiency this finding might have clinical implications.

C. Vitamin D Levels

Data concerning serum levels of $1,25(OH)_2D$ or 25-hydroxyvitamin D [25(OH)D] in psoriatic patients are conflicting. Some studies report reduced concentrations of $1,25(OH)_2D$ in patients with manifest disease [47], while others report normal levels [48]. Additionally, the coincidence of pustular psoriasis with hypocalcemia [49] and the exacerbation of psoriasis under chloroquin therapy (thereby reducing $1,25(OH)_2D$ levels via inhibition of the extrarenal 25-hydroxyvitamin D-1α-hydroxylase) are well known [50] and may give rise to speculation about vitamin D levels and severity of psoriasis.

V. CLINICAL USE OF $1,25(OH)_2D_3$ AND ITS ANALOGS IN PSORIASIS

A. Topical Use

The use of $1,25(OH)_2D_3$ and its analogs for the treatment of psoriasis resulted from two independent lines of investigation. Since psoriasis is a hyperproliferative skin disorder, it seemed reasonable that the antiproliferative effects of $1,25(OH)_2D_3$ could be used for the treatment of this disease. However, before launching clinical trials in 1985, MacLaughlin and associates reported the observation that psoriatic fibroblasts are partially resistant to the antiproliferative effects of $1,25(OH)_2D_3$ [51]. This observation prompted the authors to speculate that $1,25(OH)_2D_3$ may be effective in pharmacologic dosages for the treatment of psoriasis. The other line of investigation resulted from a clinical observation. In 1985, Morimoto and Kumahara reported that a patient, who was treated orally with 1α-hydroxyvitamin D_3 for osteoporosis, had a dramatic remission of psoriatic skin lesions [52]. Morimoto *et al*. reported a follow-up study, demonstrating that almost 80% of 17 patients with psoriasis who were treated orally with 1α-hydroxyvitamin D_3 at a dose of 1.0 μg/day for up to six months showed clinically significant improvement [53].

Numerous studies have reported that various vitamin D analogs, including $1,25(OH)_2D_3$ (calcitriol), calcipotriol (calcipotriene), tacalcitol ($1,24-(OH)_2D_3$), hexafluoro-$1,25-(OH)_2D_3$ [54], and maxacalcitol (22-oxo-$1,25-(OH)_2D_3$) are effective and safe in the topical treatment of psoriasis [55–62]. It has been shown that topical $1,25(OH)_2D_3$ is very effective and safe in the long-term treatment of psoriasis vulgaris [55] (Fig. 3). Applied twice daily topically in amounts of up to 100 g ointment (50 μg calcipotriol/g ointment) per week, calcipotriol, a synthetic analog of calcitriol, was shown to be slightly more effective in the topical treatment of psoriasis than betamethasone 17-valerate ointment [63]. Recently, efficacy of topical treatment with maxacalcitol was compared with topical calcipotriol treatment [58]. In this study, investigators' overall assessment suggested that maxacalcitol 25 μg/g applied daily may be more effective than once-daily calcipotriol (50 μg/g). It has been reported that a mild dermatitis can be seen in about 10% of patients treated with calcipotriol (50 μg/g), particularly on the face [64]. This side effect (mild dermatitis on the face) is not reported after topical treatment with $1,25(OH)_2D_3$. Allergic contact dermatitis to vitamin D analogs is very rare, however, cases with allergic contact dermatitis to other ingredients of the ointment including propylene glycol have been reported [65–67]. The most common adverse event observed in psoriasis patients treated with maxacalcitol (6–50 μg/g maxacalcitol/g ointment) was burning of the target plaque [58]. In three out of four patients that developed this side effect in one study, symptoms were severe enough to require discontinuation of the treatment [58].

A double-blind, right/left comparison, placebo-controlled evaluation demonstrated efficacy and safety

of topical treatment with hexafluoro-1,25$(OH)_2D_3$ (50 μg/g) in psoriasis patients [59]. Adverse events included mild irritation. This irritation did not necessitate discontinuation of the study medication. During the large area topical application study period, a cobblestone appearance was initially noted in a few patients. This resolved with continued therapy after 3–4 weeks. Hexafluoro-1,25$(OH)_2D_3$–treated plaques also developed very mild perilesional scales as observed with other vitamin D analogs [59]. Efficacy and safety of topical treatment with tacalcitol (4 μg/g and 20 μg/g) has been shown as well [61,62,68]. In one study [62], tacalcitol-treatment was generally well-tolerated, and there were no serious or unexpected adverse events reported. However, discontinuation of treatment as a result of skin irritation was seen in 5.9% of these patients [62]. The greatest frequency of cutaneous side-effects occurred during initial treatment and the incidence decreased markedly and the treatment was well-tolerated with continued use [62]. Recently, the results of four separate studies designed to evaluate specific local-safety parameters of vitamin D analogs including cumulative irritancy, cutaneous contact sensitization, photoallergic contact sensitization, and phototoxicity were analyzed [69]. 1,25$(OH)_2D_3$ 3 μg/g ointment was classified as nonirritant when compared to calcipotriol, tacalcitol, and white petrolatum. Petrolatum and tacalcitol were slightly irritant and calcipotriol moderately irritant. No sensitization was observed with 1,25$(OH)_2D_3$ 3 μg/g ointment. With regard to phototoxic potential, sites treated with 1,25$(OH)_2D_3$ 3 μg/g ointment or vehicle ointment were less irritated than those treated with white petrolatum or those that were untreated. Using standard photoallergenicity testing methodology, there were no skin reactions of a photoallergic nature to the 1,25$(OH)_2D_3$ [69].

Patients with psoriasis may need intermittent treatment for the rest of their lives. Vitamin D analogs have been shown not to exhibit tachyphylaxis during treatment of psoriatic lesions and can be continued indefinitely. They are effective and safe for the treatment of skin areas that are usually difficult to treat in psoriatic patients and that respond slowly. Additionally, vitamin D analogs are effective in the treatment of psoriatic skin lesions in children and in HIV-patients.

B. Oral Use

Recently, a long-term follow-up study demonstrated the efficacy and safety of oral 1,25$(OH)_2D_3$ in the potential treatment of psoriasis [70] (Fig. 5). Of the 85 patients included in that study who received oral 1,25$(OH)_2D_3$, after 36 months 88.0% had some improvement in their disease, 26.5%, 26.3%, and 25.3% had complete, moderate, and slight improvement in their disease, respectively.
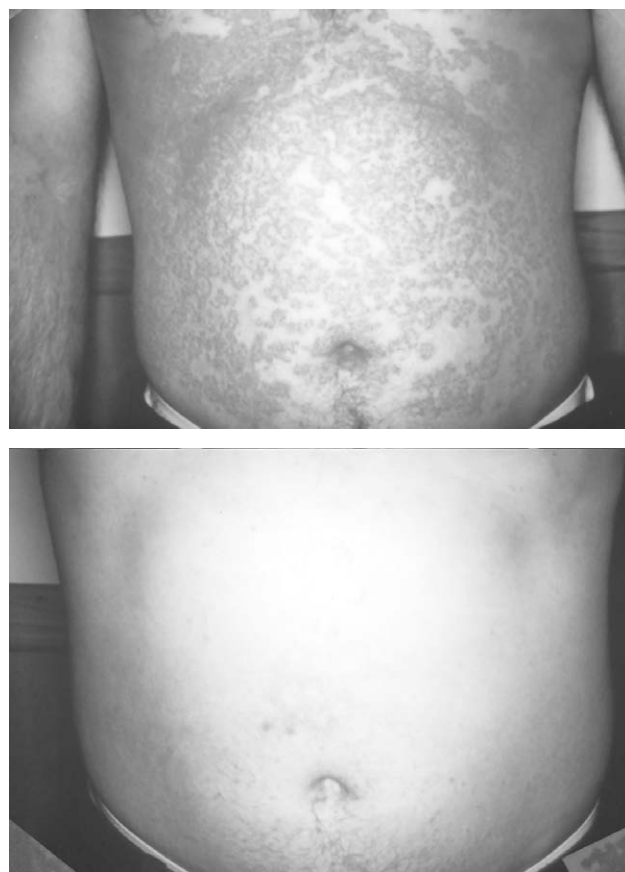

FIGURE 5 Forty-year-old male with psoriasis for 15 years. Before (**top**) and 6 months after (**bottom**) 2 μg each night of oral 1,25$(OH)_2D_3$. Reproduced with permission [70].

Serum calcium concentrations and 24 h urinary calcium excretion increased by 3.9% and 148.2%, respectively, but were not outside the normal range. Bone mineral density of these patients remained unchanged. A very important consideration for the use of orally administered calcitriol is the dosing technique. To avoid its effects on enhancing dietary calcium absorption, it is very important to provide 1,25$(OH)_2D_3$ at nighttime and with a low calcium diet. Perez *et al*. [70] showed that as a result of this dosing technique, doses of 2 μg/ to 4 μg/night are well-tolerated by psoriatic patients, although doses over 2 μg caused hypercalcemia in prostate cancer trials (Chapter 94).

C. Specific Therapies

1. TREATMENT OF SCALP PSORIASIS

Recently, a double-blind, randomized multicenter study demonstrated that calcipotriol solution is effective in the topical treatment of scalp psoriasis [71–73]. Forty-nine patients were treated twice daily over

a four-week period [71]. In the study, 60% of the patients on calcipotriol showed clearance or marked improvement vs. 17% in the placebo group. No side effects were reported.

2. Treatment of Nail Psoriasis

The occurrence of nail psoriasis has been reported in up to 50% of patients. Nails in general are very difficult to treat and respond slowly to other therapies. Up to now, there has been no consistently effective treatment for psoriatic nails. Recently, it was shown that calcipotriol ointment is effective in the treatment of nail psoriasis [74].

3. Treatment of Face and Flexures

Although the use of calcipotriol ointment is not recommended on face and skin flexures due to irritancy, most patients tolerate vitamin D analogs on these sites. Recently, it has been shown that calcitriol ointment (3 µg of calcitriol per gram of petrolatum) was found to be better-tolerated and would appear to be more effective than calcipotriol ointment (50 µg/g of petrolatum) in the treatment of psoriasis in sensitive areas [75].

4. Treatment of Skin Lesions in Children

During the last few years, it has been shown that topical application of vitamin D analogs, including $1,25(OH)_2D_3$ ointment (3 µg of calcitriol per gram of petrolatum), is an effective, safe, and reliable therapy to treat psoriatic skin lesions in children [76–78].

5. Treatment of Psoriatic Lesions in HIV-Patients

We treated an HIV-positive patient suffering from psoriatic skin lesions with topical and oral calcitriol. The patient responded well, and there was no evidence of enhancement in HIV-disease activity or alterations in the number of T lymphocytes or CD4+ and CD8+ cells (Fig. 6).

6. Combination of Vitamin D Analogs with Other Therapies

Recently, it was reported that efficacy of topical treatment with vitamin D analogs in psoriasis can be increased by combination with other therapies, including methotrexate (MTX), very low-dose oral cyclosporine (2 mg/kg/day), oral acitretin, topical dithranol, topical steroids, PUVA (psoralen plus UVA) and UV-B or narrow band UV-B phototherapy [79–88]. It has been shown that the combination of calcipotriol and MTX is safe and well-tolerated [87]. The combination resulted in lower cumulative dosages of MTX compared with MTX and vehicle. Therefore, the risk of MTX-induced side-effects is substantially decreased [87]. Addition of calcipotriol
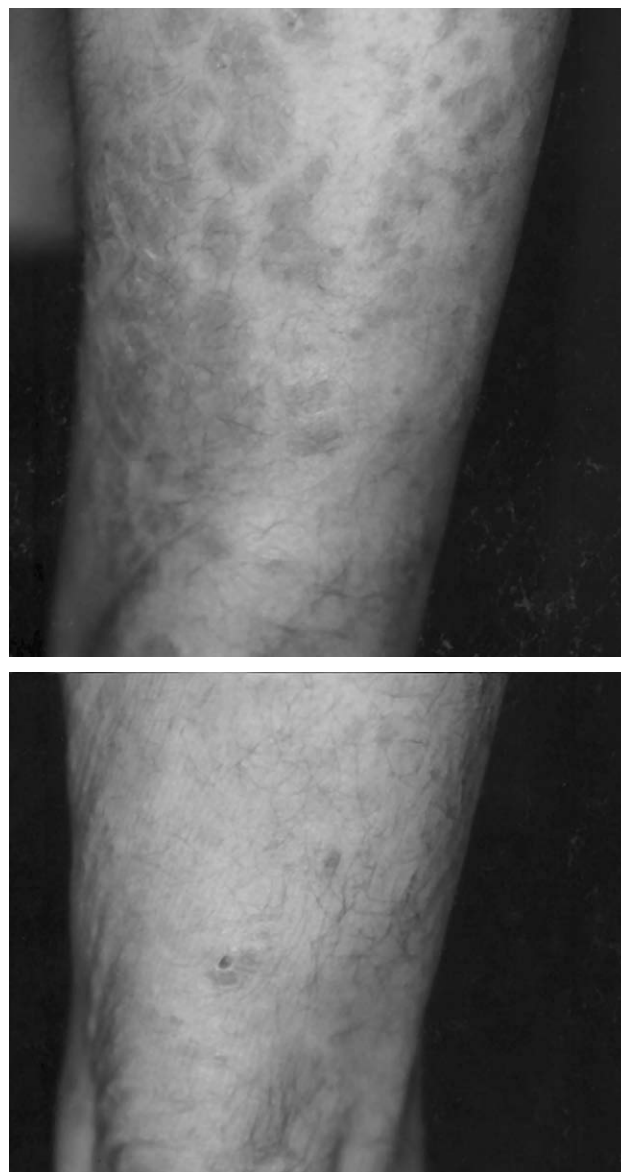

FIGURE 6   A 46-year-old man with AIDS-associated psoriasis. (**top**) before, and (**bottom**) after 3 months of oral $1,25(OH)_2D_3$ treatment.

ointment to oral application of acitretin (a vitamin A analog) was shown to produce a significantly better treatment response achieved with a lower cumulative dose of acitretin in patients with severe extensive psoriasis, as compared with the group of patients treated with oral acitretin alone [81]. The number of patients reporting adverse events was similar between the two treatment groups [81].

Complete clearing or 90% improvement in psoriasis area and severity index (PASI) was observed in 50% of patients treated with calcipotriol/cyclosporine vs. 11.8% in the placebo/cyclosporine group. No difference was

found in that study between the groups in short-time side effects.

Kragballe and co-workers reported that efficacy of topical calcipotriol treatment in psoriasis can be improved by simultaneous ultraviolet B phototherapy. Combination therapy of psoriasis with topical calcipotriol and narrow-band UVB has been shown to be very effective for the treatment of psoriatic plaques [80]. It has been shown that the combination of topical treatment with vitamin D analogs and UV radiation does not alter the tolerability or safety of therapy [88]. Vitamin D analogs may be topically applied at any time up to two hours before or immediately after UV radiation [88]. Results of a controlled, right/left study have demonstrated that pretreatment of psoriasis with the vitamin D_3 derivative tacalcitol increases the responsiveness to 311-nm ultraviolet B [89]. Additionally, it was shown that tacalcitol ointment (4 µg/g) and 0.1% tazarotene gel are both comparably effective in improving the therapeutic result of PUVA therapy in patients with chronic, plaque-type psoriasis [90]. The treatment requirements to induce complete or near complete clearing were significantly lower for both combination treatments than for PUVA monotherapy ($P < 0.01$). The median cumulative UVA dose and number of exposures were 30.6 J/cm² (95% confidence interval, CI 22.5–71.2) and 14 (95% CI 11–16) for tacalcitol plus PUVA, 32.3 J/cm² (95% CI 22.5–73.8) and 14 (95% CI 11–19) for tazarotene plus PUVA, and 37.0 J/cm² (95% CI 29.5–83.9) and 16 (95% CI 14–22) for PUVA monotherapy. No difference between the three regimens was observed with regard to duration of remission. Adverse reactions occurred more often with 0.1% tazarotene than with tacalcitol but were, in general, mild and completely reversible upon using a lower concentration of 0.05% tazarotene. It has been concluded that besides accelerating the treatment response, both agents, by virtue of their UVA dose-sparing effect, might also help to reduce possible long-term hazards of PUVA treatment. Previously, a case report described two patients treated with a combination treatment of calcipotriol and both psoralens and ultraviolet A who developed hyperpigmentation at the lesional sites where calcipotriol ointment was applied [91].

Combined topical treatment with calcipotriol ointment (50 µg/g) and betamethasone ointment was shown to be slightly more effective and caused less skin irritation than calcipotriol used twice daily [82]. Recently, a new vehicle has been created with the objective of obtaining optimal stability of both calcipotriol and betamethasone dipropionate in a combination product. Early onset of action and efficacy of a fixed combination of calcipotriol and betamethasone dipropionate in this new vehicle in the treatment of psoriasis has been reported recently [92].

VI. VITAMIN D ANALOG THERAPY IN OTHER SKIN DISEASES

A. Vitamin D and Ichthyosis

A double-blind, bilaterally paired, comparative study showed the effectiveness of topical treatment with calcipotriol ointment on congenital ichthyoses [93]. Reduction in scaling and roughness on the calcipotriol-treated side was seen in all patients with lamellar ichthyosis and bullous ichthyotic erythroderma of Brocq. The only patient treated with Comel-Netherton syndrome showed mild improvement, while the only patient suffering from ichthyosis bullosa of Siemens that was treated with calcipotriol did not show any change in severity on the calcipotriol-treated as compared to the vehicle-treated side. Recently, it has been reported that topical tacalcitol therapy was ineffective against ichthyoses without epidermal hyperproliferation but with retentive hyperkeratosis, including X-linked ichthyosis (XLI), ichthyosis vulgaris (IV), and acquired ichthyosis [94].

B. Vitamin D Analogs and Scleroderma

Previous findings point to the efficacy of vitamin D analogs for the treatment of scleroderma. Humbert *et al*. [95] reported that oral administration of 1.0–2.5 µg/d calcitriol improves skin involvement, probably via inhibition of fibroblast proliferation and dermal collagen deposition.

C. Vitamin D Analogs and Skin Cancer

In vitro studies have demonstrated strong antiproliferative and prodifferentiating effects of vitamin D analogs in many VDR-expressing tumor cell lines, including malignant melanoma, squamous cell carcinoma, and leukemic cells [34,96]. *In vivo* studies showed that active vitamin D analogs reduced proliferation and tumor progression of epithelial tumors in rats [97], as well as other cancers [98,99] (see Section IX of this book). Little is known regarding the effects of calcitriol on the formation of metastases in patients with malignant melanoma or squamous cell carcinoma of the skin.

D. Vitamin D Analogs and Other Skin Diseases

A number of case reports demonstrated positive effects of topical treatment with vitamin D analogs in a variety of skin diseases such as transient acantholytic

dermatosis (Grovers disease), inflammatory linear verrucous epidermal naevus (ILVEN), disseminated superficial actinic parakeratosis, pityriasis rubra pilaris, epidermolytic palmoplantar keratoderma of Vorner, confluent and reticulated papillomatosis (Gougerot-Carteaud syndrome), and Sjögren-Larsson syndrome [100, rev. in 101]. These promising observations will have to be further evaluated in clinical trials.

VII. PERSPECTIVES FOR THE DEVELOPMENT OF NEW VITAMIN D ANALOGS WITH LESS CALCEMIC ACTIVITY FOR THE TREATMENT OF HYPERPROLIFERATIVE SKIN DISORDERS

The use of vitamin D analogs in dermatology and other medical fields has been limited, since serious side effects, mainly on calcium metabolism, have occurred at the supraphysiological doses needed to achieve clinical efficacy. The evaluation of new vitamin D analogs with strong immunosuppressive, antiproliferative, and differentiating effects but only marginal effects on calcium metabolism will introduce important new therapies for the treatment of various skin and other diseases. The goal to create new vitamin D analogs with selective biological activity and minimal undesirable side effects has not yet been reached, but recent findings are promising. This subject is extensively discussed in Section VIII of this book.

Calcipotriol, a vitamin D analog with similar VDR binding properties compared to calcitriol but low affinity for the vitamin D–binding protein (DBP), is effective, safe, and approved for the topical treatment of psoriasis [56,63]. *In vivo* studies in rats showed that effects of calcipotriol on calcium metabolism are 100–200-fold lower compared to calcitriol, while *in vitro* effects on proliferation and differentiation on human keratinocytes are comparable [102]. These differential effects are probably caused by the different pharmacokinetic profiles of calcipotriol and calcitriol (different affinity for DBP). Serum half-life in rats of these vitamin D analogs was shown to be 4 min after treatment with calcipotriol in contrast to 15 min after treatment with calcitriol [102]. However, one has to emphasize that the calcium studies comparing calcitriol and calcipotriol were done *in vivo* while most studies analyzing proliferation or differentiation were done *in vitro*.

A different approach to create new vitamin D analogs that are effective in the topical treatment of hyperproliferative or inflammatory skin diseases is the goal to create new synthetic compounds with a high degree of dissociation that are metabolized in the skin and therefore exert only few systemic side effects. New analogs of vitamin D, obtained by a combination of the 20-methyl modification and removal of the C-19 methylene with side-chain modification including triple-bond at C-23 [103] or 2β-substituted $1,25(OH)_2D_3$ [104], are promising candidates.

Another interesting approach to enhance the concentration of calcitriol locally in the skin without obtaining systemic side effects are attempts to specifically inhibit the activity of vitamin D metabolizing enzymes, (i.e., 24-hydroxylase for calcitriol) that are present in the skin and responsible for the catabolism of calcitriol [105]. It is known that various active drugs, including other steroidal hormones and cytochrome P450 inhibitors such as ketoconazole, inhibit the activity of 24-hydroxylase in the skin [106]. It may be possible to enhance the concentration of endogenous calcitriol locally in the skin by the topical application of these compounds without obtaining systemic side effects. We speculate that the therapeutic effects of various antimycotic compounds including ketoconazole in the treatment of seborrheic dermatitis may at least in part be due to this mechanism.

It is known that VDR requires several accessory proteins for efficient binding to vitamin D response elements in promoter regions of target genes and induction of transactivation [107]. As a consequence, different vitamin D analogs, depending on their chemical structure, may have different affinities for the various homo- or heterodimers of VDR and nuclear cofactors including RXR-α [108]. The synthesis of new vitamin D analogs that activate different vitamin D signaling pathways may lead to the introduction of new therapeutics for the topical or oral treatment of various skin diseases. These new drugs may induce strong effects on target cell proliferation and differentiation in the skin or the immune system, but only marginal effects on calcium metabolism.

Another approach to enhance the therapeutic effects of orally or topically administered calcitriol may be the combination with synergistic drugs. The recent discovery of different vitamin D signaling pathways that are determined and regulated by cofactors of VDR including RXR-α and their corresponding ligands, suggests that 9-cis RA or all-trans RA may act synergistically with vitamin D analogs to induce VDR-mediated transactivation and regulate the transcriptional activity of distinct gene networks. Little is known about the effects of the combined application of vitamin D and vitamin A analogs under physiological conditions *in vivo*. This combination may selectively enhance or block different biological effects of vitamin D analogs

that are mediated by different vitamin D signaling pathways.

In conclusion, it can be speculated that new vitamin D analogs will introduce new alternatives for the treatment of various skin disorders. If the final goal can be reached to create strong antiproliferative and antiinflammatory vitamin D analogs with only little calcemic activity, these new agents may herald a new era in dermatologic therapy, that possibly can be compared with the introduction of synthetic corticosteroids or retinoids. These new drugs, which activate selective vitamin D signaling pathways but exert only little calcemic activity, will also be effective in the systemic treatment of various malignancies and other diseases in addition to skin diseases. However, since skin diseases can also be treated by topical application of drugs, additional strategies are available to develop analogs with improved safety and efficacy for the treatment of psoriasis and other skin disorders.

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Muscles and Falls

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I. INTRODUCTION

For many years, myopathy has been considered part of the osteomalacic symptom complex [1–6], occasionally seen in patients suffering from osteomalacic bone disease caused by severe vitamin D deficiency. In agreement with this notion, the first term suggested for vitamin D deficiency-related myopathy was "osteomalacic myopathy" [5,7]. Recently, however, myopathy has been shown to be a prominent, and common symptom of vitamin D deficiency. Furthermore, new data also indicate that severely impaired muscle function may be present, even before biochemical signs of bone disease develops [8]. Myopathy has been demonstrated in vitamin D deficient "fallers," even without signs of secondary hyperparathyroidism [9]. Consequently, we suggest the use of the term "hypovitaminosis D myopathy" (HDM) for this condition.

The interest in HDM has varied over the years. In the seventies, several publications focused on myopathy in relation to vitamin D deficiency [6,10–17], whereas research on HDM was limited to experimental studies of nongenomic effects of 1,25(OH)$_2$D$_3$ in the eighties and early nineties. These studies are reviewed elsewhere in this book (Chapter 55).

In 1992 Chapuy et al. [18] presented a 43% reduction in hip fracture rate in elderly women treated with 800 IU cholecalciferol and 1200 mg calcium for 18 months (see Chapter 66). The rapid reduction in hip fractures demonstrated in this study was difficult to explain by vitamin D-induced improvements in bone mass only, and thus suggested that improvement of muscle tone and coordination after vitamin D might play a role. Ever since this pivotal study, the interest in clinical studies of the muscular effects of vitamin D has been growing. Several studies have demonstrated, that large proportions of hip fracture patients are vitamin D deficient [19–21], and these findings led several groups to suggest that the epidemic of hip fractures among the elderly could at least partly be explained by falls secondary to vitamin D deficiency-related myopathy. Consequently, hip fractures might be prevented by vitamin D supplementation. In support of this hypothesis, Bischoff et al. [9] recently reported a 49% reduction of falls among elderly treated with 800 IU cholecalciferol and 1200 mg calcium for 3 months.

II. HYPOVITAMINOSIS D MYOPATHY (HDM): SYMPTOMS AND SIGNS

HDM is often overlooked and misdiagnosed [22]. In this context, it is important to understand that the decrease in muscle strength is a continuous and gradual process, whereas the loss of functional ability is quantal. This means that the patients can have a considerable loss of muscle strength before they complain of muscle weakness. Their main complaint would most likely be fatigue. The patients do not complain of muscle weakness until they are unable to walk or experience difficulties rising from a squatting position unaided. Secondly, many of the symptoms of HDM are rather diffuse (pain, fatigue, weakness, paresthesias, etc.) and thus often lead to alternative diagnoses such as rheumatic disease, polymyalgia, psychoneurotic disorders, fibromyalgia, malignant diseases, etc.

In a recent attempt to evaluate HDM-related symptoms among 65 patients with different degrees of vitamin D deficiency and 22 controls, a graded scale questionnaire was used [23,24]. The most prominent

symptoms were diffuse muscle pain of the proximal muscles of the lower limb, proximal arm muscles and shoulder muscles, followed by deep bone pain, fatigue, difficulty in ascending a staircase/carrying loads, peripheral paresthesias, muscle cramps, and joint pain [5,12,22]. The symptoms all correlated significantly to serum levels of 25OHD, whereas no significant correlation to $1,25(OH)_2D$ levels could be detected [23].

The waddling gait classically associated with HDM [2,4,5] is only seen in severe cases of vitamin D deficiency. The typical "faller" or hip fracture patient will often present muscular symptoms, but most clinicians will attribute such symptoms to aging. However, if patients are asked specifically for symptoms of HDM, they will usually present them. Moreover, muscle strength measured by a dynamometer will often be reduced. HDM typically affects proximal muscle groups, particularly the weight-bearing antigravity muscles of the lower limb [8].

As mentioned above, the symptoms of HDM are rather nonspecific. Thus, assessment of serum 25OHD and PTH is obligatory when the diagnosis HDM

is suspected. Presence of secondary hyperparathyroidism or low levels of 25OHD will strongly support the diagnosis of HDM. Measurement of serum calcium, alkaline phosphatase, and $1,25(OH)_2D$ are of limited value in the diagnosis of HDM, as values can be normal even when severe HDM symptoms are present [8].

III. MUSCLE PHYSIOLOGY IN RELATION TO HDM

A detailed review of muscle ultra structure and physiology is beyond the scope of this chapter. However, in order to understand the effects of vitamin D on striated muscle, a short introduction is warranted. Striated muscle cells consist of bundles of myofibrils enveloped in the cell membrane/sarcolemma (see Fig. 1). The myofibrils are surrounded by the sarcoplasmatic reticulum (SR), the calcium reservoir of the cell. The diameter of muscle fibers varies (10–100 μm) [25,26]. From the sarcolemma, tubular invaginations (the T-tubules) penetrate deeply into the cell making
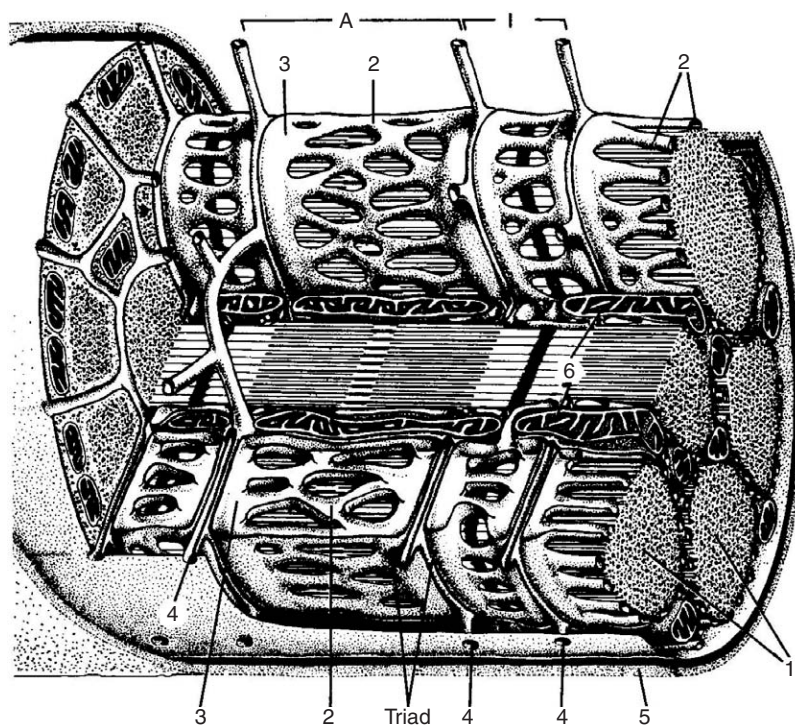

FIGURE 1   Skeletal muscle. A single muscle fiber surrounded by its sarcolemma (5). The sarcoplasmatic reticulum (2) with its terminal cisternae (3) surrounds each myofibril (1) which is a build up of thick and thin filaments. The T-tubules (4) are invaginations of the cell membrane (sarcolemma) penetrating deep into the muscle fiber. The arrays of mitochondria are situated among the myofibrils (6). Reproduced by permission from Krstic RV: Ultrastructure of the mammalian cell. Springer-Verlag, 1979.

a network around the myofibrils, where they are located very near to the terminal cisternae of SR in the so called "triads." The network of the T-tubules and the SR are so extensive that a 100 μm diameter fiber with a cell surface of 1 cm² has a T-tubular surface of 7 cm² and a SR surface of 135 cm² [27]. Calcium release from the SR will always take place within 1 μm distance from the binding site on troponin C [28]. The myofibrils consist of regularly arranged myosin
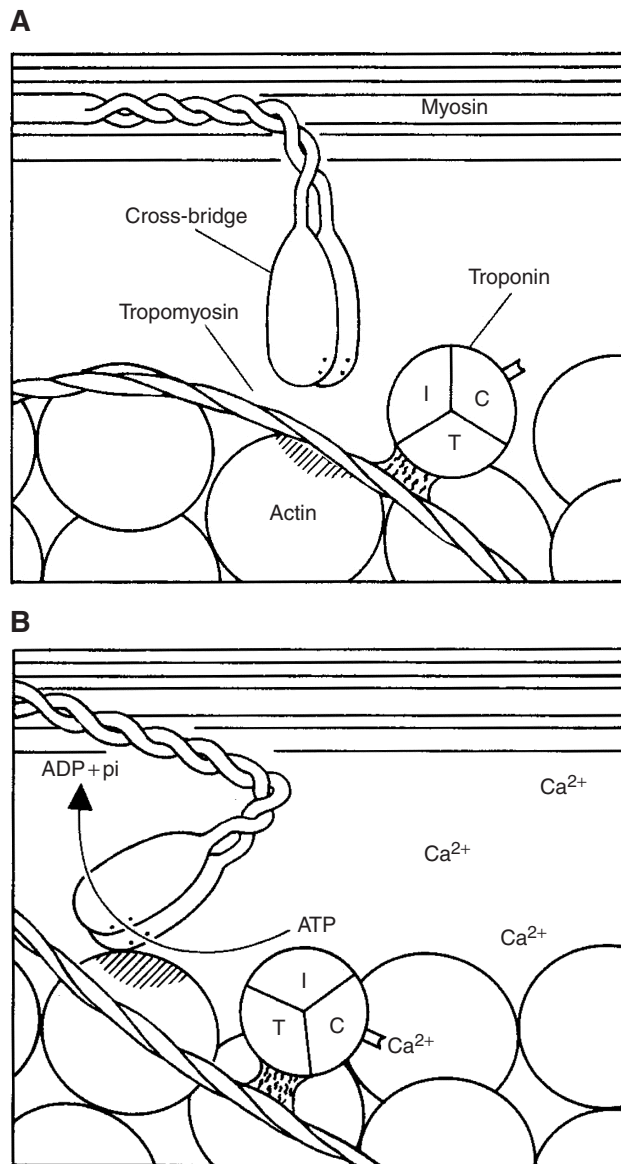
A

B

FIGURE 2   Myosin heads extending from the thick filaments reach towards binding sites on the thin actin filaments. In relaxation the binding sites are covered by troponin. With binding of Ca++ to troponin C, a conformation change occurs allowing the myosin head to bind to its actin binding site, ATP is consumed and contraction is initiated. Modified by permission from N Engl J Med 1975;293:1184.

and actin filaments, giving the muscle the characteristic striated appearance under the microscope. A myofibril comprises 100–400 filaments and has a diameter of 1 μm [25].

The filaments consist of three components: actin, tropomyosin, and troponin (see Fig. 2). The myosin molecules of the thick filament are arranged in a helix with myosin heads projecting in a 45° angle from the longitudinal direction of the filament [25–27,29]. The thin filaments possess a binding site for the myosin head, which under relaxation is covered by troponin. Troponin is made up of three subunits: troponin I (binds to actin), troponin T (binds to tropomyosin), and troponin C (has strong affinity for Ca++).

A muscle contraction is initiated by a depolarization reaching the muscle from the motor-end-plate via the T-tubules. The depolarization results in liberation of calcium from the terminal cisternae of the SR through activation of voltage-dependent calcium release channels. As the Ca++ concentration rises, Ca++ will bind to troponin C and this results in a conformational change of the actin filament, which allows the myosin head to bind to the binding site (see Fig. 2). In the presence of Mg++ and under consumption of ATP the myosin head bends 45° and subsequently moves along the actin filament (10 nm per bend) [29]. Relaxation occurs as Ca++ is lowered again (see Fig. 3).

A. Calcium Homeostasis in Muscle Cells

As the calcium ion is essential for muscle function, maintenance of calcium homeostasis is a highly prioritized process in the muscle cell. The Ca++ concentration in the cytosol of the resting muscle is very low (10^{-7}–10^{-8} mol/l), 10,000 times lower than extracellular levels (2×10^{-3} mol/l). An energy dependent pump, in the form of Ca-ATPase, maintains this gradient. This pump is localized both in the outer cell membrane (sarcolemma) pumping Ca++ out of the cell, as well as in the sarcoplasmatic membrane pumping Ca++ into the sarcoplasmatic reticulum (SR). The content of Ca-ATPase of the SR is much higher than in the sarcolemma, which makes SR the most important organelle for regulation of intracellular calcium homeostasis. In the SR, a calcium-binding protein (calsequestrin) keeps the concentration of free Ca++ at a level of 0.5×10^{-3} mol/l. Calsequestrin has the capacity of binding 43 mol Ca++ per mol protein. If all Ca++ were free, the concentration in SR could be as high as 20×10^{-3} mol/l [29]. The calsequestrin is bound to the calcium release channels of the SR, and may be important in the regulation of Ca++ release from the SR [30,31].
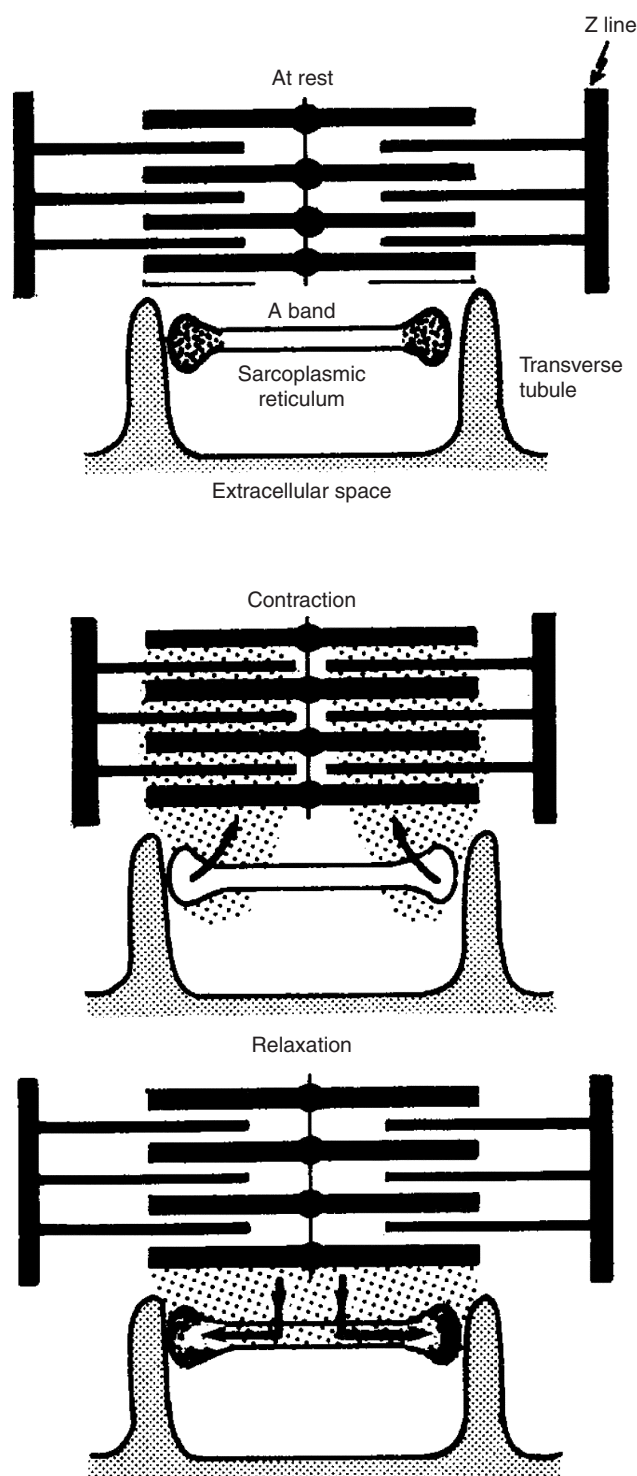
FIGURE 3   Muscle contraction—relaxation. During rest, calcium is stored in the terminal cisternae of the sarcoplasmatic reticulum. The action potential spreads via the T-tubules and releases calcium and thus initiates the contraction. Calcium is pumped into the sarcoplasmatic reticulum, and the muscle relaxes. Modified with permission from N Engl J Med 1971;285:31.

During muscle contraction, the Ca^{++} level in the cytosol can increase to 2×10^{-4} mol/l. Relaxation of the muscle occurs when Ca^{++} has been pumped back into the SR. If stimulation stops, cytosolic calcium levels can be normalized within 1/20 second. Thus Ca-ATPase plays a pivotal role in the regulation of calcium homeostasis and function of muscle cells.

B. Energy Supply

Muscles demand a steady energy supply as ATP is consumed both during muscle contraction and muscle relaxation. During muscle contraction, the available pool of ATP in the cell is consumed within a few seconds. New ATP is produced by oxidative phosphorylation of glucose, fatty acid, and amino acids through the respiratory chain, and further degradation to CO_2 and H_2O through the O_2-demanding citric acid circle. Muscles also have the capacity of glycogenesis as storage of energy for later use. Under anaerobic conditions, glucose can undergo anaerobic glycolysis leading to the formation of lactic acid and ATP. For periods of high energy demand, ATP is stored as phosphocreatine, which rapidly yields ATP via the reaction: phosphocreatine + ADP $\leftrightarrows$ creatine + ATP.

C. Fiber Types

Based on histochemical staining, muscle fibers are divided into three types: I, type IIa, and type IIb. The three fiber types differ in their energy metabolism. Type I fibers possess the capacity for oxidative metabolism making them more fatigue resistant. Type IIb fibers are solely dependent on fast energy supply from ATP, creatinephosphate, and from glycolysis of glucose to lactic acid. Type IIa fibers have both oxidative and glycolytic capacity, making them more fatigue-resistant than type IIb. Type I fibers are smaller and weaker than type II; they are more easily excited and are recruited before the type II fibers. Type II fibers are innervated by big motor-neurones and are big fibers suitable for precise and powerful movements, whereas type I are more suitable for maintaining a basal muscle tonus [29,32]. In agreement with type II fibers being the more powerful, the terminal cisternae of type II fibers contain up to 7 times more Ca-ATPase than the type I fibers [33,34]. The fiber composition differs between the different muscle groups of the body. A distal muscle, such as soleus, is mainly built of type I fibers, whereas a proximal muscle like quadriceps has a high percentage of type II fibers [25].

IV. EXPERIMENTAL STUDIES ON THE EFFECTS OF VITAMIN D ON STRIATED MUSCLE

A. Genomic and Nongenomic Effects of Vitamin D on Striated Muscle

Striated muscle contain vitamin D receptors (VDR) [35–38], and vitamin D has been shown to stimulate the synthesis of several important muscle proteins (troponin C, actin) in the sarcoplasmatic reticulum and the inner membrane of mitochondria [17,39,40].

In addition to the classical genomic effects of vitamin D, a number of nongenomic effects have been identified [41–43]. The nongenomic effects are mediated through binding of vitamin D to membrane receptors resulting in formation of intracellular second messengers, which elicit rapid regulation intracellular enzyme activity and ion-pump systems. These effects are extensively reviewed elsewhere in this book (Chapter 55).

B. Animal and Cell Culture Studies

The importance of vitamin D for muscle function was firmly established, when Curry *et al.* [44] published their study in Nature in 1974. They showed that the ATP-dependent Ca^{++} uptake of isolated vesicles of SR was reduced in muscle from vitamin D–depleted rabbits, when compared to vitamin D-repleted animals. They further reported that the administration of three oral doses of vitamin D_3 (100 IU) to the vitamin D–depleted animals normalized the Ca^{++}-uptake.

A few years later, [14] Rodman and Baker [45] published another important study. They used an *in vivo* model of vitamin D–depleted and –repleted rats for electrophysiological investigations of the muscle kinetics of the soleus muscle (see Fig. 4). In this model, vitamin D–depleted animals exhibited prolonged time-to-peak contractile tensions and prolonged relaxation half-lives. Again, this could be normalized by a few days of vitamin D treatment prior to muscle testing. Moreover, they demonstrated that the phenomenon was independent of serum levels of Ca^{++} and phosphate.
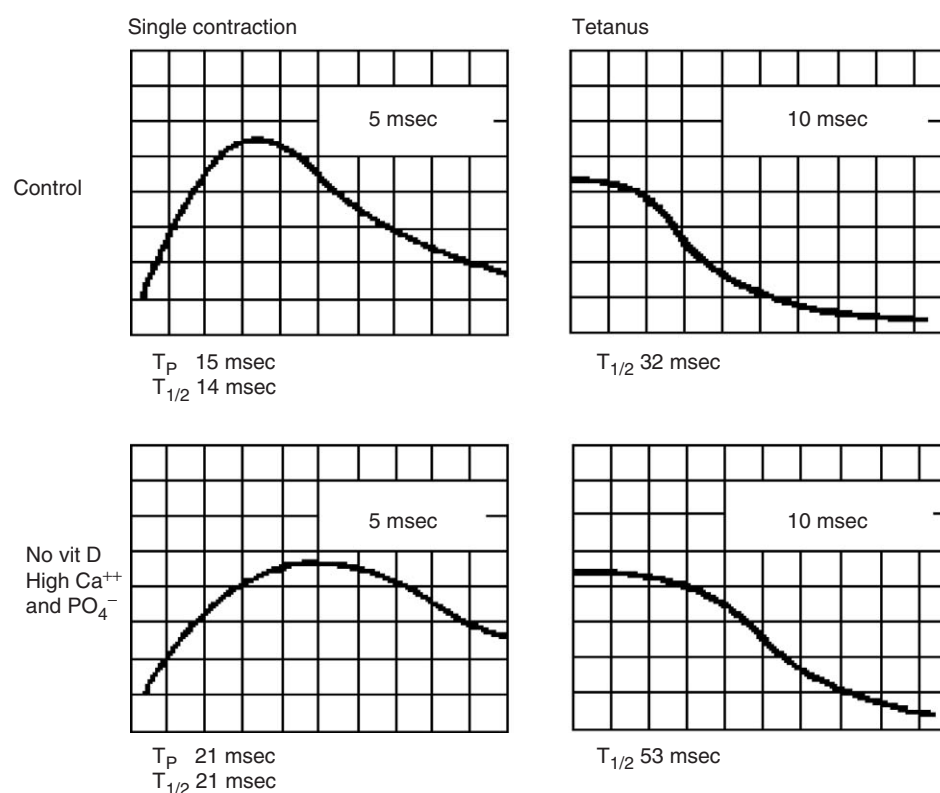

FIGURE 4   Contraction and relaxation response in vitamin D–depleted and –repleted rats. Reproduced by permission from Rodman and Baker [45], Kidney International 1978;**13**:189–193.

Birge and Haddad [16] studied the effect of 25OHD on muscle in an *in vitro* model. They found that 25OHD increased the intracellular content of ATP and phosphate and increased protein synthesis. Vitamin D_3 and $1,25(OH)_2D$ were found to have no effects on these mechanisms.

The active transport of Ca^{++} into SR by Ca-ATPase is $1,25(OH)_2D$–dependent [46–48]. The activity of the pump seems to be regulated by $1,25(OH)_2D$–stimulated phosphorylation of proteins in the SR-membrane [41]. Furthermore, stereological studies [49] have shown that vitamin D–deficient rats exhibit reduced SR volume and increased mitochondria volume. These changes are also reversed by treatment with $1,25(OH)_2D$.

The myofibrillar content of actin and troponin C is reduced in vitamin D–depleted animals [17,39] and normalized by vitamin D treatment. This normalization is most likely an action of 25OHD, rather than $1,25(OH)_2D$ [17].

In two studies [39,50], the content of Ca-ATPase in vitamin D–depleted animals was investigated. It was found to be normal and unchanged by vitamin D treatment. Also the histochemical and electron microscopic appearance of muscles from vitamin D–depleted animals in the two studies was completely normal. These studies, however, do not rule out an effect of vitamin D on Ca-ATPase synthesis. Type II fibers, usually described as being atrophic in vitamin D–depleted individuals, show a much higher content of Ca-ATPase than other fiber types.

Vitamin D_3 supplementation of vitamin D–depleted chicks enhances [3H]-leucine labeling of proteins of the inner mitochondrial membrane by 50%, of sarcoplasmatic proteins by 20%, and contractile proteins by 10% [40]. These results indicate that vitamin D exerts important effects on the protein synthesis in muscle. The pronounced effect on the mitochondrial proteins suggests a regulatory effect on energy metabolism.
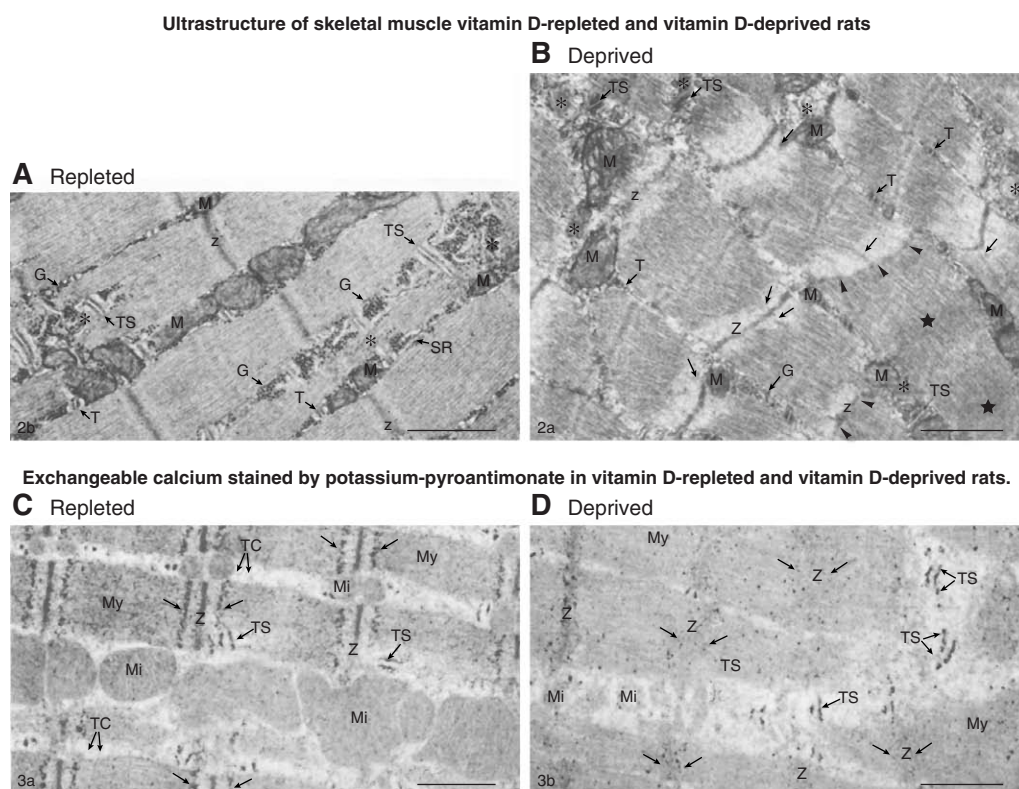

FIGURE 5   Ultrastructure of skeletal muscle in vitamin D–repleted (upper left (a)) and vitamin D–depleted (upper right (b)) rats. Notice the disturbed architecture of the muscle in vitamin D–depleted rats. Some of the sarcomeres fail to relax (arrow heads). Z: Z-line, SR: Sarcoplasmic Reticulum, M: Mitochondria, G: Glycogen, T: Triad, TS: T-System. In the lower part of the figure, the functional pool of protein bound exchangeable calcium has been visualized by potassium-pyroantimonate staining. In vitamin D–repleted rats (lower left (c)) high concentration of calcium appears at the T-system (TS) and the terminal cisternae (TC) of the sarcoplasmic reticulum. Irregular dispersed calcium is seen along the myofibrils (My). The calcium staining is less intensive and more irregular in vitamin D–depleted rats (lower right (d)). Reproduced from Toury *et al.* 1990 Biology of the Cell. **69**:179–189 with permission from Elsevier.

Toury *et al.* [51] investigated the calcium content in subcellular fractions of vitamin D–depleted and –repleted rats. In all subcellular fractions except the cytosol, they found lower Ca^{++} contents in vitamin D–depleted compared to vitamin D–repleted animals. In the cytosol, the calcium content was higher in vitamin D–depleted animals compared to vitamin D–repleted, indicating lower activity of the Ca-ATPase. In electron microscopic studies of muscle biopsies, exchangeable calcium was visualized with pyroantimonate-osmium (see Fig. 5). Vitamin D–replete animals revealed much more intensive staining of the areas around the terminal cisternae of the SR, the T-tubules, and at the active sites of the myofibrils. Thus, the net effect of vitamin D on intracellular calcium homeostasis seems to be augmentation of intracellular calcium fluxes and an increased calcium pool available for muscle contraction.

V. CLINICAL STUDIES ON HYPOVITAMINOSIS D MYOPATHY

A. Biopsy Studies

Muscle biopsies obtained in severely vitamin D–deficient patients revealed selective atrophy of type II muscle fibers with enlarged interfibrillar spaces and infiltration of fat, increased fibrosis, and glycogen granules [6,11–15,52]. Mitochondria were irregular in size and shape. These findings are not specific for HDM, but can be seen in a number of other endocrine myopathies [53]. However, in neuropathic atrophy, both type I and type II fibers are affected [25]. In immobilization atrophy, type I fibers are primarily affected [25]. Sato *et al.* [54] recently investigated muscle biopsies from hip fracture patients. The patients were divided into vitamin D–sufficient (25OHD > 39 nmol/l, n = 20) and vitamin D–deficient (serum 25OHD < 39 nmol/l, n = 22). In the vitamin D–deficient group, type II fibers were significantly smaller (15.4 ± 4.2 µm) than in the vitamin D–sufficient group (38.7 ± 8.1 µm) ($p < 0.0001$). Furthermore, in the vitamin D–deficient group, type II fiber diameters correlated with serum levels of 25OHD ($r = 0.714$, $p = 0.001$). No such correlation was demonstrable in the vitamin D–sufficient group.

Eight patients with vitamin D deficiency (25OHD = 7.0 ± 0.7 nmol/l) underwent muscle biopsies of the vastus lateralis muscle before and after three months of vitamin D treatment. The Ca-ATPase content increased significantly during treatment (before 3.4 ± 0.4, after 4.7 ± 0.2 nmol/g wet wt, $p < 0.02$) [23]. This increase is most likely explained by regeneration of type II fibers, which have a higher overall Ca-ATPase content.

The Ca-ATPase content correlated to serum levels of 25OHD ($r = 0.5$, $p < 0.05$) but not to $1,25(OH)_2D$ or PTH.

During sudden movements (e.g., correction of postural imbalance), the fast and strong type II fibers are the first to be recruited to avoid falling [32]. Thus, the selective type II fiber atrophy seen in HDM may be closely associated with the tendency to fall in vitamin D–deficient individuals [23,55,56].

B. Clinical Studies on Muscle Function

In 1975 Skaria *et al.* [6] reported that 25 of 30 patients with osteomalacia exhibited an abnormal electromyogram, with signs of both myopathy and reduced nerve conduction velocity. Fourteen patients were followed during vitamin D treatment for several months. All but one showed improvement in their electromyograms, whereas no improvement in nerve conduction could be detected.

In a prospective study Young *et al.* [10] followed the progress in muscle function in 12 osteomalacic patients during vitamin D treatment. They used an isometric dynamometer model for measurement of quadriceps muscle strength, and obtained muscle biopsies from the lateral vastus muscle. A significant improvement in both muscle strength and muscle biopsies was demonstrated after three months of treatment, but full restitution of the muscles required treatment for 6 to 12 months.

Several studies have shown clinical improvement in muscle strength and function following vitamin D therapy. In two studies α-calcidiol 0.5 µg/d given to frail vitamin D–deficient elderly individuals, improved knee extension strength, walking speed [57], and "time taken to dress" [15].

In 349 elderly individuals (age > 70 years) Mowe *et al.* [58] demonstrated a significant correlation between handgrip-strength and serum levels of 25OHD ($r = 0.22$, $p < 0.001$) and an inverse correlation between tendency to fall and 25OHD ($r = 0.30$, $p < 0.05$). These findings are in agreement with those of Bischoff *et al.* [59], who investigated leg extension power in relation to serum levels of vitamin D metabolites in 103 women and 216 men (65–95 years). In men, muscle power correlated to 25OHD ($r = 0.24$, $p < 0.0004$) and $1,25(OH)_2D$ ($r = 0.14$, $p < 0.045$). In women, no significant correlation to 25OHD was found. However, only 12% of the women exhibited low 25OHD levels (< 30 nmol/l).

Using a scoring system for daily living activities Gloth *et al.* [60] were able to show improvement during vitamin D treatment, whereas Corless *et al.* [61] found no effect of supplementation with ergocalciferol

(9000 IU) to frail old patients. A high prevalence of severe comorbidities in the study group, however, most likely influenced the evaluation of functional performance. The presence of severe comorbidity probably also explains the negative results in the study of Verreault *et al.* [62].

Boonen *et al.* [63] investigated the correlation between muscle function and serum levels of $1,25(OH)_2D$ in 245 elderly women (70–90 years). No correlation could be demonstrated. 25OHD levels were not reported in this study. Grady *et al.* [64] studied the effect of daily supplementation with 0.5 μg calcitriol to 98 elderly (age >69 years). No effect on muscle function was found. However, the study group displayed a normal vitamin D status (25OHD > 60 nmol/l), and thus no HDM would be expected. Both studies are confounded by the age related loss of muscle strength (1.5% pr year) reported by McComas [32], which seems to be obligatory and nonpreventable by vitamin D.

In veiled Arab women living in Denmark (n=55) ($25OHD = 6.7 \pm 0.6$ nmol/l), Glerup *et al.* [8,65] demonstrated a 34% reduction in muscle power determined by voluntary knee extension (MVC), when compared to controls (N=22) with normal vitamin D levels. A series of ergocalciferol injections (100,000 IU weekly for one month followed by 100,000 IU monthly for six months) and a daily supplement of 1200 mg calcium were given. MVC increased by 13% after three months and by 24% after six months ($p < 0.02$). Electrical stimulation of the quadriceps muscle was also performed. The resulting single-twitch measurements (see Fig. 6) allowed estimation of muscle kinetics. Using this experimental setup, the maximal force production is derived from the slope of the ascending limb of the curve, while the slope of the descending limb of the curve expresses the maximal relaxation rate. The vitamin D–deficient group exhibited slower and weaker muscles compared to controls. After six months of treatment, however, muscle kinetics of the quadriceps was completely normalized. These findings corroborate the findings of Rodman and Baker [45] in rats. Glerup *et al.* also demonstrated that HDM precedes biochemical signs of bone involvement [8]. Further, MVC correlated significantly with serum levels of 25OHD (r=0.34, $p < 0.01$) and PTH (r=−0.33, $p<0.001$), but not with $1,25(OH)_2D$ (r=−0.14, NS). In a multivariate regression analysis, only 25OHD was found to be significant. Decreased muscle power could be detected when serum levels of 25OHD were below 60 nmol/l (see Fig. 7). Some of the participants underwent investigation with ^{31}P-MR-spectroscopy [66] in order to determine the effects of vitamin D status on energy metabolism. The muscular content of energy rich phosphocreatine and inorganic phosphate were
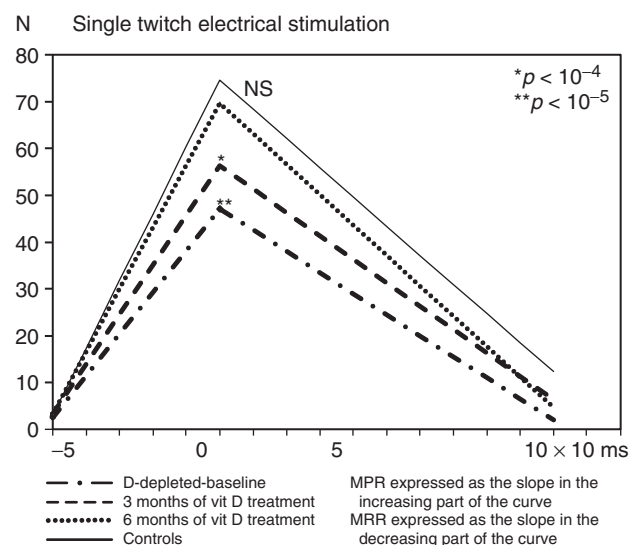

FIGURE 6 Single-twitch electrical stimulation of the quadriceps muscle was performed in an isometric dynamometer model with the knee in 90° bowed position. 55 vitamin D–deficient patients ($25OHD = 6.7 \pm 0.6$ nmol/l) had performed measurement before and after 3 and 6 months of high-dose vitamin D treatment. The results were compared with those of 22 matched controls with normal vitamin D level. The ascending part of the curve expresses the maximal production rate of the muscle. The descending part expresses the maximal relaxation rate. Reproduced with permission from Calcified Tissue International 2000;66:419–424. [8].
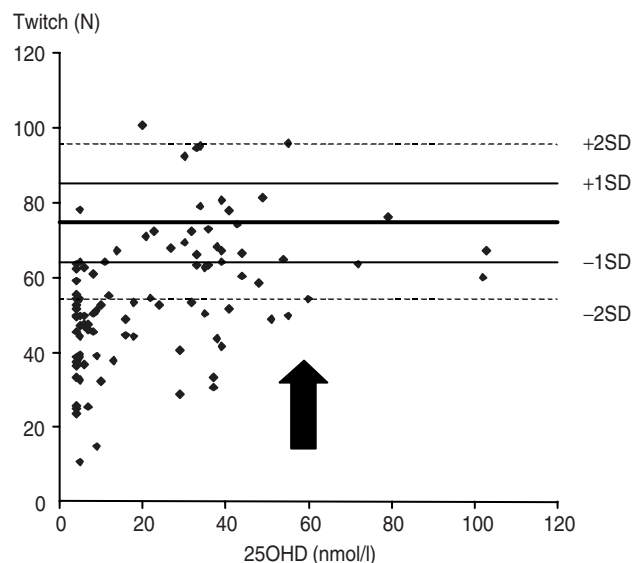

FIGURE 7 In single-twitch electrical stimulation of the quadriceps muscle, decreased muscle function could be detected at a level of 25-OHD below 60 nmol/l. The solid and dotted lines indicate the range of normal values (mean 74.6 nmol/l ± 2 SD) of controls with normal levels of 25OHD (47.1 ± 4.6 nmol/l) [8].

determined during rest, work, and recovery after work. At rest, vitamin D–deficient patients exhibited significant lower levels of phosphocreatine and inorganic phosphate than controls. During work, the decline in phosphocreatine and increase in inorganic phosphate was higher in vitamin D–deficient patients. Also, the recovery time after contraction was significantly longer in vitamin D–deficient patients than controls (56 s vs. 36 s, $p < 0.05$). After three months of vitamin D treatment all measurements were normalized.

The susceptibility of individuals to vitamin D deficiency may also be genetically determined. Geusens et al. [67] recently reported effects of VDR polymorphisms on muscle function. In nonobese women they found a 23% higher quadriceps strength in genotype bb compared to genotype BB ($p < 0.01$).

VI. CLINICAL STUDIES ON THE RISK OF FALLS AND VITAMIN D STATUS

A. Vitamin D and Fracture Risk

As mentioned in the introduction to this chapter, the study by Chapuy et al. [18] inspired many investigators to perform studies on the muscular effects of vitamin D. In a group of 3270 elderly (84 ± 6 years) nursing home residents, they demonstrated a 43% reduction in hip fracture incidence after 18 months of treatment with 800 IU cholecalciferol and 1200 mg calcium daily. This reduction in hip fracture incidence was somewhat surprising since the change in BMD was more modest. BMD of the proximal femur increased by 2.7% in the vitamin D–treated group compared to a 4.6% decrease in the placebo group. Re-evaluation after three years continued to show a 25% reduction in hip fracture incidence. The obvious question was: could the results be partly explained by improvement in muscle function and consequently reduced risk of falls?

The relations between muscle function, risk of falls, and vitamin D status have recently been extensively reviewed by Jansen et al. [68] and Pfeifer et al. [69]. They found that 90% of all hip fractures involve a fall [70,71]. Fractures caused by falls occur in about 5% of elderly persons every year, with the hip being involved in 1–2% [72–74].

The hypothesis that vitamin D supplementation may reduce the risk of falls and thus fractures has gained further support from several studies. Dawson-Hughes et al. [75] found a 58% reduction in nonvertebral fractures in 389 elderly (age > 65 years) treated with daily supplementation of 700 IU cholecalciferol and 500 mg calcium. Heikenheimo et al. [76] gave an annual injection of ergocalciferol (150,000–300,000 IU) to 621 elderly (age > 74 years) resulting in a significant reduction in fractures of the upper extremities. Lips et al. [77], on the other hand, found no effect on the incidence of hip or peripheral fractures in a 3.5 years study of 2578 elderly supplied with 400 IU cholecalciferol daily. Meyer et al. [78] gave the same dose of cholecalciferol to 569 elderly nursing home residents for two years. Again no fracture-preventing effect was seen when compared to matched controls. The negative results of the last two studies are most likely explained by the low dose of vitamin D used in these subjects. Furthermore, no calcium supplementation was given. This notion is further corroborated by the fact that the patients in the Lips study still showed signs of secondary hyperparathyroidism at study end-point [77].

Seasonal variation of hip fracture incidence [79,80], as well as a higher incidence of hip fractures at higher latitudes [80,81], has been described. This is most likely caused by seasonal variations in sunlight exposure and resulting serum levels of 25OHD [82]. It is less likely that changes in bone due to seasonal variation in 25OHD could result in increased fracture incidence [83]. In Europe, 36% of men and 47% of women aged > 70 years exhibit wintertime levels of 25OHD < 30 nmol/l [84]. Serum levels of 25OHD have been shown by Chapuy et al. [85] to correlate with latitude (r=−0.7, $p < 0.01$). More pronounced seasonal variations in 25OHD at higher latitudes might be a contributing factor to the increase in hip fracture incidence during wintertime at high latitude.

Exposure to sunlight is quite essential in maintenance of normal vitamin D status and in the prophylaxis against HDM. Normally the skin supplies the body with 80–100% of its requirements of vitamin D [86]. If sunlight exposure of the skin is limited, however, 25OHD will decrease and HDM may develop. A study of veiled Caucasian women living in Denmark showed that lack of direct sunlight exposure required a daily supply of 800 IU vitamin D_3 in order to maintain normal vitamin D status [65]. This is in agreement with the study of Holick [86], who investigated vitamin D levels in submariners. A supply of more than 600 IU vitamin D_3 was necessary to maintain normal levels of vitamin D during the absence of sunlight for three months. In elderly fallers Dhesi et al. [87] described a relationship between 25OHD levels and the number of times per week the patient went outside. Thus, homebound frail old people have a high risk for development of HDM making them even more immobile. They get less sunlight exposure, and moreover their age-dependent skin atrophy further reduces the ability to produce vitamin D, despite ample sunlight exposure [86]. These data lead to the following conclusion: all individuals

at risk of HDM, especially the elderly (>65 years) who are not regularly exposed to sunlight, should be given a daily supplement of at least 800 IU vitamin D_3 in order to avoid HDM.

B. Vitamin D and Risk of Falls

33% of elderly people experience at least one fall per year [72–74]. Mowe *et al.* [58] found lower levels of 25OHD among fallers compared to nonfallers with an inverse correlation between serum levels of 25OHD and the risk of falls ($r = -0.27$, $p < 0.001$). This is in agreement with the findings of Stein *et al.* [56], who described a correlation between secondary hyperparathyroidism and the risk of falls. In a study of 4251 elderly Australian women living in residential care (age >84 years), Flicker *et al.* [88] recently identified low serum levels of 25OHD as a major risk factor for falls (hazard risk ratio 0.64, $p < 0.004$).

In a "falls clinic" in London, to which patients with at least one fall within the last 8 weeks, were referred, Dhesi *et al.* [87,89] described severe hypovitaminosis D (25OHD < 30 nmol/l) in 31.8% and moderate hypovitaminosis (25OHD < 50 nmol/l) in 72.8%. Patients with low levels of 25OHD (< 30 nmol/l) displayed significantly impaired psychomotor function measured by a performance test (AFPT), postural sway, choice reaction time (CRT), and isometric quadriceps strength measurement. In a multivariate analysis, 25OHD was identified as an independent variable for AFPT, CRT, and body sway. For quadriceps strength, PTH was found to be an independent variable.

Pfeifer *et al.* [90] investigated the effect on body sway and fall incidence in 148 women (aged 74 ± 1 year) treated with either a daily dose of 1200 mg calcium in combination with 800 IU cholecalciferol or 1200 mg of calcium alone. After one year, a 9% decrease in body sway was seen in the vitamin D–treated group compared to the group treated with calcium only ($p = 0.043$). The number of falls per subject per year was 0.45 in the calcium-only group compared to 0.24 in the vitamin D–treated group ($p = 0.034$).

Bischoff *et al.* [9] treated 122 elderly women (mean age 83.3 years) with either 1200 mg calcium in combination with 800 IU cholecalciferol (Cal + D) or calcium only (Cal). Musculoskeletal function was measured by knee extension and flexion strength, grip strength, and timed "up & go" test. After three months, a significant improvement in musculoskeletal function was found in the Cal + D group ($p < 0.0094$). The risk of falling was reduced by 49% when comparing the Cal + D to the Cal group ($p < 0.01$) (see Fig. 8).

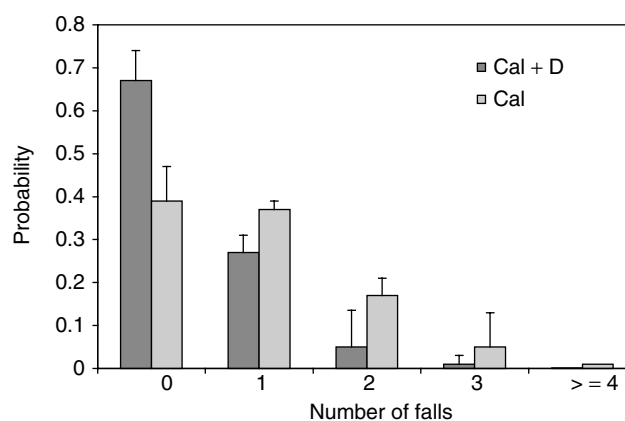The data presented above lend strong support to the hypothesis, that it is vitamin D supplementation more

FIGURE 8 Number of falls and the effect of treatment with 800 IU cholecalciferol + 1200 mg calcium daily (Cal + D) versus calcium alone (Cal). Adjusted probabilities and SEs for having zero, one, or multiple falls for subjects in both treatment groups. SEs are calculated by taking 1 SD above and below the mean rate of falls and calculating the resulting Poisson probabilities. Adjustments have been performed for length of observation in the treatment period, previous falls in pretreatment period, a person who fell in the pretreatment period, age, and baseline 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D. Reproduced from Bischoff HA, *et al.* J Bone Miner Res 2003;18:343–351 with permission of the American Society for Bone and Mineral Research.

than calcium supplementation that protects against falls. The analysis of these studies suggests that a daily dose of at least 800 IU vitamin D_3 should be given in order to ensure vitamin D sufficiency and significant improvement in muscle function and reduction in the incidence of falls.

VII. IS HDM CAUSED BY LOW LEVELS OF 25OHD, 1,25(OH)$_2$D OR ELEVATED PTH?

From a theoretical point of view, the most likely effector associated with HDM should be 1,25(OH)$_2$D. VDR has been identified in muscle [35–38] and 1,25(OH)$_2$D shows the highest affinity for the receptor. In agreement with this theory, experimental research has almost exclusively focused on the muscular effects of 1,25(OH)$_2$D. Clinical experience, however, indicates that complaints associated with HDM correlate to serum levels of 25OHD and not to 1,25(OH)$_2$D. The lack of correlation to 1,25(OH)$_2$D levels might be due to increased renal 1-α-hydroxylase activity caused by secondary hyperparathyroidism. PTH, on the other hand, might also exert direct effects on muscle. In the following sections the three "candidates" will be discussed separately.