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## Synergistic induction of human cathelicidin antimicrobial peptide gene expression by vitamin D and stilbenoids

Chunxiao Guo<sup>#1,2</sup>, Brian Sinnott<sup>#1,2</sup>, Brenda Niu<sup>4</sup>, Malcolm B. Lowry<sup>1,3</sup>, Mary L. Fantacone<sup>1,2</sup>, and Adrian F. Gombart<sup>1,2</sup>

<sup>1</sup>Linus Pauling Institute, Oregon State University, Corvallis, Oregon 97331

<sup>2</sup>Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

<sup>3</sup>Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

<sup>4</sup>School of Medicine, Oregon Health Sciences University, Portland, Oregon 97239

# These authors contributed equally to this work.

### Abstract

**Scope**—The cathelicidin antimicrobial peptide (CAMP) gene is induced by 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>), lithocholic acid, curcumin, nicotinamide and butyrate. Discovering additional small molecules that regulate its expression will identify new molecular mechanisms involved in CAMP regulation and increase understanding of how diet and nutrition can improve immune function.

**Methods and results**—We discovered that two stilbenoids, resveratrol and pterostilbene, induced CAMP promoter-luciferase expression. Synergistic activation was observed when either stilbenoid was combined with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. Both stilbenoids increased CAMP mRNA and protein levels in the monocyte cell line U937 and synergy was observed in both U937 and the keratinocyte cell line, HaCaT. Inhibition of resveratrol targets sirtuin-1, cyclic AMP production and the c-Jun N-terminal, phosphoinositide 3 and AMP-activated kinases did not block induction of CAMP by resveratrol or synergy with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. Nevertheless, inhibition of the extracellular signal-regulated 1/2 and p38 mitogen-activated protein kinases, increased CAMP gene expression in combination with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> suggesting that inhibition of these kinases by resveratrol may explain, in part, its synergy with vitamin D.

**Conclusions**—Our findings demonstrate for the first time that stilbenoid compounds may have the potential to boost the innate immune response by increasing CAMP gene expression particularly in combination with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>.

### Keywords

cathelicidin antimicrobial peptide; innate immunity; resveratrol; stilbenoid; vitamin D receptor

## 1 - Introduction

Modulating the expression of endogenous antimicrobial peptides (AMPs) or proteins provides a viable approach for boosting the innate immune response as bacterial pathogens are less likely to develop resistance to AMPs [1]. Nutrients consumed in our food or through

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Corresponding author: Adrian F. Gombart, Associate Professor, Linus Pauling Institute, 307 Linus Pauling Science Center, Corvallis, OR 97331 USA; adrian.gombart@oregonstate.edu, tel. 541-737-8018, fax 541-737-5077.

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dietary supplements may provide a practical means to improve immune function by increasing the expression of AMPs [2]. The human cathelicidin antimicrobial peptide (*CAMP*) gene is an ideal candidate for increasing barrier defense as the peptide is effective at killing a wide range of bacteria and is expressed by both immune and epithelial cells [3].

The expression of the human *CAMP* gene is induced by  $1\alpha,25(\text{OH})_2\text{D}_3$ , lithocholic acid, butyrate, and vitamin B3 [4–10]. The first two compounds induce expression by acting as ligands for the vitamin D receptor (VDR) which binds to the *CAMP* gene promoter [4, 5], butyrate treatment increases PU.1 and CREB1 recruitment to the *CAMP* promoter [8, 11] and vitamin B3 increases C/EBP $\epsilon$  binding to the *CAMP* promoter [10]. Based on a mammalian two-hybrid study, it was proposed that polyunsaturated fatty acids (PUFAs) may act as low affinity ligands like lithocholic acid and thus regulate VDR-target gene expression [12]. In this same study, curcumin was identified as novel ligand for the VDR in colon cancer cell and shown to induce CYP24A1 gene expression. Recently, we demonstrated that curcumin modestly induced *CAMP* gene expression through a VDR-independent pathway in myeloid and colon cells, but PUFAs did not [13].

In addition to the VDR, it was shown that the primary bile salt chenodeoxycholic acid (CDCA) induced the expression of the human *CAMP* gene in a biliary carcinoma cell line through the farnesoid X receptor (FXR) [14]. It was proposed that CDCA increased binding of FXR to the *CAMP* promoter and activated gene expression, but the binding site for FXR was not identified [14]. With the possibility of additional VDR ligands and other steroid hormone receptors binding to the VDRE in the *CAMP* promoter, we hypothesized that additional small molecules may modulate *CAMP* gene expression. The discovery of additional small-molecule regulators of the *CAMP* gene would increase our knowledge of the biologically relevant pathways involved in regulating *CAMP* gene expression and could lead to better understanding of how diet and nutrition affect immune function and/or the development of therapeutically useful natural compounds to boost the innate immune response.

To identify new compounds that regulate *CAMP* gene expression, the NIH Clinical Collection of 446 molecules that are being used in human clinical trials was screened in U937 myeloid cells transfected with the human cathelicidin promoter sequence cloned into the two-step transcriptional activator (TSTA) luciferase reporter construct [15]. We discovered that both resveratrol and pterostilbene activated the *CAMP* promoter and endogenous *CAMP* gene expression was induced in both myeloid and keratinocyte cell lines by either stilbenoid. Furthermore, when pterostilbene or resveratrol was combined with  $1\alpha,25(\text{OH})_2\text{D}_3$  or its analogs there was a significant synergistic increase in *CAMP* gene expression above levels for cells treated with either active vitamin D or the stilbenoid alone.

## 2 - Materials and Methods

### 2.1 - Cell Culture

The myeloid leukemia cell line U937 and the keratinocyte cell line HaCaT were grown in RPMI 1640 or DMEM, respectively, supplemented with 10% FBS and antibiotics (100 units penicillin/streptomycin; Life Technologies, Carlsbad, CA). Cells were treated with various combinations of compounds at concentrations and times indicated in the figure legends. Resveratrol,  $1,25(\text{OH})_2\text{D}_3$  and sirtinol were purchased from Sigma-Aldrich Corporation (St. Louis, MO); pterostilbene was purchased from VWR (Radnor, PA). The AMP kinase (AMPK) inhibitor BML-275 and adenylate cyclase inhibitor 2',3'-dideoxyadenosine (2',3'-DDA) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The kinase inhibitors for ERK1/2 (AZD6244), p38 MAP kinase (SB203580), c-Jun kinase (SP600125) and PI3 kinase (LY294022) were all purchased from Selleck Chemicals (Houston, TX).

## 2.2 – Small Molecule Library Screen

A portion of the human *CAMP* promoter (nucleotides –693 to +14) [5] was cloned into the two-step transcriptional amplification vector that expresses firefly luciferase (FFL) and was kindly provided by Michael Carey, University of California at Los Angeles (Fig. 1) [15]. U937 ( $5 \times 10^7$ ) cells were transfected with 5  $\mu\text{g}$  of the TSTA-CAMP-FFL and phTKRL that expresses *Renilla* luciferase (RL; Promega Corporation, Madison, WI) for normalization of FFL expression. Transfections were performed using the Neon System (Tip-100, 1400v, 30ms, 1 pulse) as described by the manufacturer (Life Technologies) and cells were incubated with RPMI1640 medium supplemented with 10% FBS and no antibiotics. At 8 h post transfection, the cells were evenly seeded into four 96-well plates with antibiotics and treated with control compounds (DMSO, ethanol or  $1\alpha,25(\text{OH})_2\text{D}_3$ ) or test compounds from the NIH Clinical Collection (NCC-003) (BioFocus DPI, Inc, Little Chesterford, UK) at a 10  $\mu\text{M}$  concentration. At 24 h post-transfection, Dual-Glo Luciferase assays (Promega Corporation) were performed as instructed by the manufacturer and quantified using a SpectraMAXL luminometer (Molecular Devices, Sunnyvale, CA). Compounds that induced *CAMP* reporter activity were tested against the promoter-less TSTA vector to verify that induction was dependent on the presence of the *CAMP* promoter.

## 2.3 - RNA isolation and quantitative real-time PCR (QRT-PCR)

Total RNA from  $2 \times 10^6$  U937 cells was prepared with Trizol as described by the manufacturer (Life Technologies). All cDNAs were synthesized from 2  $\mu\text{g}$  of RNA using Superscript III reverse transcriptase as described by the manufacturer (Life Technologies). The cDNAs were analyzed by Q-PCR using Taqman probes specific for human *CAMP*, *CYP24A1*,  $\beta$ -actin and 18S rRNA as described previously [13]. Reactions were performed in triplicate for each sample, normalized to 18S rRNA and the fold change was calculated using  $\Delta\Delta\text{CT}$  values (treatment versus untreated) or the ratio of target gene/housekeeping gene (18S rRNA) was determined ( $\text{ratio} = 2^{-(\text{Ct}_{\text{target}} - \text{Ct}_{18\text{S}})}$ ). To determine statistical significance between two different means, a Student's T-test was performed ( $p < 0.05$ ). To compare more than two means, ANOVA was performed followed by a Fisher's least significant difference procedure ( $p < 0.05$ ).

## 2.4 - Flow Cytometry

U937 cells were treated with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  with or without 10  $\mu\text{M}$  pterostilbene or resveratrol for 24 h. Cells were fixed, permeabilized, blocked and stained with primary and secondary or secondary antibody alone as described previously [13]. The primary antibody for hCAP-18 was rabbit anti-hCAP18, kindly provided by Niels Borregaard [16], and the secondary antibody was a Dylight 649 Fab' 2 donkey anti-rabbit (Jackson ImmunoResearch, Pike West Grove, PA, USA). Fluorescence activated cell sorting (FACS) was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analyzed by BD CellQuest™ Pro software (BD Biosciences).

## 3 - Results

### 3.1 – Chemical Library Screen

To screen chemical libraries for small molecule activators of *CAMP* gene expression, a two-step transcriptional activator (TSTA) reporter construct [15] containing 710 bp of the upstream promoter region (–696 to +14) of the *CAMP* gene was generated (Fig. 1). This strategy was utilized to augment the activity of the human *CAMP* promoter [5]. Rather than directly inducing the firefly luciferase gene (one-step activation), the *CAMP* promoter induces expression of a GAL4DBD-vp16 fusion protein, a very potent transcriptional activator, that binds to five GAL4 binding site repeats in the plasmid and thus driving

expression of the firefly luciferase gene (two-step activation, Fig. 1). Using this reporter construct resulted in a 30–40-fold increase in absolute firefly relative light units (RLUs) as compared with the one-step construct (data not shown).

The expression of the *CAMP* gene is induced in the U937 myeloid leukemia cell line when it is treated with  $1\alpha,25(\text{OH})_2\text{D}_3$ , LCA, butyrate or curcumin [5, 13, 17]; therefore, we selected this cell line for transfection with the TSTA-*CAMP* construct and the small molecule library screen. To verify that this system would detect activators of the *CAMP* gene, U937 cells were transfected with TSTA-*CAMP* and treated with ethanol or DMSO (both negative controls) or 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  (positive control). Ethanol and DMSO did not activate the TSTA-*CAMP* construct, but  $1\alpha,25(\text{OH})_2\text{D}_3$  increased FFL activity by 3–4-fold. A Z-factor of 0.86 was calculated from three independent experiments indicating that the system would be robust enough to detect activators of the *CAMP* gene (data not shown).

The NIH Clinical Collection was screened and compounds that induced the TSTA-*CAMP* promoter construct 2-fold or greater compared to the DMSO control, without significantly decreasing RL activity, were retested in triplicate. Candidate compounds that consistently activated the TSTA-*CAMP* construct were tested in triplicate on U937 cells transfected with a promoter-less TSTA vector to exclude those compounds that non-specifically activated the backbone of the vector (data not shown). The NIH Clinical Collection compounds were also tested in combination with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  to identify small molecules that could cooperatively induce *CAMP* together with  $1\alpha,25(\text{OH})_2\text{D}_3$ . Three compounds that passed all of the criteria for candidate activators, calcipitriene, resveratrol and pterostilbene, were used in subsequent experiments. Calcipitriene is a synthetic derivative or analog of  $1\alpha,25(\text{OH})_2\text{D}_3$  while resveratrol and pterostilbene belong to the stilbenoid class of compounds which are believed to have numerous health benefits. The identification of calcipitriene was not surprising because it, like  $1\alpha,25(\text{OH})_2\text{D}_3$ , is a known VDR ligand and would be expected to induce *CAMP* gene expression. Activation by both VDR ligands demonstrated that the TSTA-FFL assay was robust enough to identify bona fide inducers of the *CAMP* gene.

### 3.2 – Induction of endogenous *CAMP* gene expression by candidate compounds

As a secondary screen, we tested the novel ability of resveratrol and pterostilbene to increase endogenous *CAMP* mRNA expression in cell culture. *CAMP* gene expression was consistently induced 2–4 fold in U937 cells treated with 10  $\mu\text{M}$  resveratrol or pterostilbene as compared to controls (Fig. 2A). Furthermore, combining either pterostilbene or resveratrol (10  $\mu\text{M}$ ) with  $1\alpha,25(\text{OH})_2\text{D}_3$  (10 nM) induced *CAMP* levels about 3-fold higher than  $1\alpha,25(\text{OH})_2\text{D}_3$  alone (Fig. 2B & C).

To determine if resveratrol specifically modulated expression of the *CAMP* gene or vitamin D target genes in general, we examined the response of another VDR target gene, CYP24A1, and a non-VDR target gene,  $\beta$ -actin (Supporting Information Fig. 1).  $1\alpha,25(\text{OH})_2\text{D}_3$  strongly induced CYP24A1 mRNA expression, but resveratrol did not (Supporting Information Fig. 1A). In addition, a combinatorial induction was not observed with resveratrol and  $1\alpha,25(\text{OH})_2\text{D}_3$  (Supporting Information Fig. 1A). The expression of  $\beta$ -actin was not induced by either  $1\alpha,25(\text{OH})_2\text{D}_3$ , resveratrol or a combination of both (Supporting Information Fig. 1B). Taken together, the data suggest that resveratrol primarily modulates *CAMP* gene expression and that it is not due to a non-specific transcriptional effect.

Human *CAMP* gene expression is induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  in keratinocytes [4, 6]. To determine if the stilbenoids would also induce *CAMP* in keratinocytes, HaCat cells were treated with resveratrol at 10  $\mu\text{M}$  or  $1\alpha,25(\text{OH})_2\text{D}_3$  at 10 nM alone or a combination of both.

There was no significant increase in *CAMP* expression in cells treated with resveratrol alone when compared to the untreated control (Fig. 3). Cells treated with  $1\alpha,25(\text{OH})_2\text{D}_3$  showed a small increase in *CAMP* expression; however, in combination with resveratrol there was an approximately three-fold increase over  $1\alpha,25(\text{OH})_2\text{D}_3$  alone (Fig. 3).

### 3.3 – *CAMP* Protein Expression

To determine if stilbenoids induced *CAMP* protein (hCAP18) levels, intracellular staining and FACS for hCAP18 was used to determine changes in protein expression (Fig. 4). As expected, U937 cells treated with  $1\alpha,25(\text{OH})_2\text{D}_3$  (1 nM) for 24 h (Fig. 4 B and D, solid curves) showed a significant shift to the right in the population's mean fluorescent intensity compared with untreated cells (Fig. 4 A and C) indicating induction of hCAP18. A modest shift was observed in cells treated with either resveratrol or pterostilbene (10  $\mu\text{M}$ ) without  $1\alpha,25(\text{OH})_2\text{D}_3$  indicating that both stilbenoids induced hCAP18 protein expression (Fig. 4 A and C, dashed or dotted curves versus solid curves). Cells incubated with either resveratrol or pterostilbene (10  $\mu\text{M}$ ) together with  $1\alpha,25(\text{OH})_2\text{D}_3$  (1 nM) showed increased hCAP18 protein expression with mean fluorescent intensities higher than those with either compound alone (Fig. 4 B and D, dashed or dotted curves versus solid curves). These results were consistent with the levels of induction of *CAMP* mRNA observed in U937 cells.

### 3.4 – Mechanism of Induction of *CAMP* by Stilbenoids

The molecular targets that mediate the effects of resveratrol are numerous and include sirtuins, cyclo- and lipoxygenases, reductases, protein kinases and transcription factors [18]. We tested several potential resveratrol targets to determine the molecular mechanism by which it increased *CAMP* gene expression.

**Activation of Sirt1**—The metabolic effects of resveratrol are tied to its ability to indirectly activate Sirt1 *in vivo* [19–22]. To determine if activation of Sirt1 was involved in the induction of *CAMP* gene expression, we treated cells with the Sirt1 inhibitor sirtinol [23]. Pterostilbene and resveratrol induced *CAMP* gene expression to similar levels in both untreated and sirtinol-treated U937 cells and sirtinol did not interfere with the synergy of  $1\alpha,25(\text{OH})_2\text{D}_3$  when combined with either stilbenoid (Fig. 5). Furthermore, NAM, another Sirt1 inhibitor, had no effect on *CAMP* gene expression in U937 cells (data not shown). Taken together, the data do not support a role for Sirt1 activation in the induction of the *CAMP* gene by either stilbenoid.

**Activation of cAMP signaling**—Resveratrol increases cAMP levels by inhibiting cAMP-degrading phosphodiesterases (PDEs) ultimately leading to the activation of the CamKK $\beta$ -AMPK pathway [24]. This pathway activates both PGC-1 $\alpha$  and Sirt1 and may explain the metabolic effects of resveratrol [24]. cAMP signaling is very complex and numerous other transcription factors are activated including the cAMP responsive element binding protein 1 (CREB1) [25]. cAMP signaling induces *CAMP* gene expression in mucosal epithelial cells via activation of the CREB1 and activator protein-1 (AP-1) transcription factors [11]. To determine if an increase of cAMP levels mediated the induction of *CAMP* by resveratrol, we pretreated U937 cells with the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (2',3'-DDA) to block the production of cAMP, but *CAMP* induction by resveratrol was not blocked (Fig. 6). Furthermore, cells treated with the PDE inhibitor rolipram, which mimics resveratrol by increasing cAMP levels, did not increase cathelicidin expression (data not shown) nor did stimulating cAMP production with forskolin (data not shown). Taken together these data do not support a role for increased cAMP levels in mediating the induction of *CAMP* expression in U937 monocytic cells by resveratrol or pterostilbene.



**Modulation of Erk1/2, p38 MAPK, JNK, PI3K and AMPK pathways**—Resveratrol modulates the MAPK, PI3K/AkT and AMPK signaling pathways [18]. To determine if one or more of these pathways is involved in the action of resveratrol on the induction of *CAMP* gene expression, we treated U937 cells with inhibitors of these kinases and determined the effect they had on *CAMP* induction with or without  $1\alpha,25(\text{OH})_2\text{D}_3$ . Numerous studies in different cell culture systems have demonstrated that resveratrol inhibits MAPK activity [26–28]. In U937 cells treated with the MAPK inhibitors AZD6244 (ERK1/2), SB203580 (p38 MAPK) and SP600125 (JNK), none of the inhibitors alone or in combination with resveratrol induced *CAMP* gene expression (Fig. 7A, Untreated) nor did they enhance or impair induction of the *CAMP* gene in a statistically significant manner (Fig. 7A, RSV). In combination with  $1\alpha,25(\text{OH})_2\text{D}_3$ , ERK1/2 and p38 MAPK inhibitors increased *CAMP* expression about 50–70% higher than  $1\alpha,25(\text{OH})_2\text{D}_3$  alone (Fig. 7A, 1,25D3). However, neither was as effective as resveratrol which increased *CAMP* expression >200% above  $1\alpha,25(\text{OH})_2\text{D}_3$  alone (Fig. 7A, 1,25D3) and inhibition of JNK did not affect *CAMP* induction by  $1\alpha,25(\text{OH})_2\text{D}_3$ . Inhibition of ERK1/2, p38 MAPK or JNK did not block the synergy observed with the combination of resveratrol and  $1,25(\text{OH})_2\text{D}_3$  and, in fact, *CAMP* levels were increased above those seen with the combination alone (Fig. 7A, RSV + 1,25D3). These increases were likely due to the effect of these inhibitors on the induction by  $1\alpha,25(\text{OH})_2\text{D}_3$ .

Resveratrol inhibits PI3K activity [29] and so we tested the effect of PI3K inhibition on induction of the *CAMP* gene by  $1\alpha,25(\text{OH})_2\text{D}_3$ . Induction of *CAMP* by  $1\alpha,25(\text{OH})_2\text{D}_3$  alone or in combination with resveratrol was inhibited by the PI3K inhibitor LY294002, but the synergy of  $1\alpha,25(\text{OH})_2\text{D}_3$  and resveratrol was still maintained (Fig. 7A, 1,25D3 vs RSV + 1,25D3). The inhibition of VDR target genes by PI3K inhibition was described previously and suggests that the overall reduction in *CAMP* expression is due to the effect of LY294002 on the vitamin D receptor [30, 31].

Resveratrol activates the AMPK pathway [32–34]; therefore, we tested the effect of AMPK inhibition on induction of the *CAMP* gene by  $1\alpha,25(\text{OH})_2\text{D}_3$ . The AMPK inhibitor BML-275 had no statistically significant effect on the ability of  $1\alpha,25(\text{OH})_2\text{D}_3$  to induce *CAMP* expression nor was the synergy with resveratrol affected by BML-275 (Fig. 7B).

Taken together, the data suggests that the inhibition of ERK1/2 and p38 MAPK by resveratrol may contribute to the enhanced expression of the *CAMP* gene observed with the combination of resveratrol and  $1\alpha,25(\text{OH})_2\text{D}_3$ , but that modulation of JNK, PI3K and AMPK activities by resveratrol do not play a role in the synergy observed between  $1\alpha,25(\text{OH})_2\text{D}_3$  and resveratrol.

### 3.5 – Combinatorial induction of *CAMP* gene expression by stilbenoids and $1\alpha,25(\text{OH})_2\text{D}_3$ analogs

Synthetic analogs of  $1\alpha,25(\text{OH})_2\text{D}_3$  are used clinically because they have a similar or higher affinity for the VDR, but display significantly less activity in regulating calcium metabolism and causing hypercalcemia as does  $1\alpha,25(\text{OH})_2\text{D}_3$  [35]. We tested whether a combination of stilbenoid with calcipotriene (Dovonex, Leo Pharma, Inc., Parsippany, NJ), an analog used topically to treat plaque psoriasis, and paricalcitol (Zemplar, Abbott Laboratories, Abbott Park, IL), another analog used to prevent or treat secondary hyperparathyroidism associated with chronic renal failure, would induce *CAMP* expression in U937 cells (Fig. 8). Both resveratrol and pterostilbene induced *CAMP* mRNA levels three-to-20-fold higher than paricalcitol alone (Fig. 8A) and four-to-eight-fold higher than calcipotriene alone (Fig. 8B). These data demonstrate that both stilbenoids synergistically activate *CAMP* gene expression with vitamin D analogs.

## 4 – Discussion

Screening of the NIH Clinical Collection of 446 compounds led to the novel discovery of two stilbenoids that induce the human *CAMP* gene. Although the induction of *CAMP* by resveratrol and pterostilbene was modest, they synergistically induced *CAMP* gene expression when combined with  $1\alpha,25(\text{OH})_2\text{D}_3$ . This synergy was observed in both monocyte and keratinocyte cell lines. The only other bona fide inducer identified in the collection was calcipotriene, a  $1\alpha,25(\text{OH})_2\text{D}_3$  analog.

Resveratrol has numerous well-documented health benefits; however, its mechanisms of action remain unclear because direct molecular targets of resveratrol are numerous and difficult to identify [18]. We tested the potential role for several molecular targets in mediating the effects of resveratrol on vitamin D induction of *CAMP* gene expression. This included activation of Sirt1 and cAMP production as well as the inhibition of MAPK, PI3K and AMPK activities. These pathways do not appear to be involved in the synergy that we observe, but the inhibition of ERK1/2 and p38 MAPK enhanced  $1\alpha,25(\text{OH})_2\text{D}_3$  induction of *CAMP* suggesting that the effect of resveratrol on *CAMP* expression may be due, in part, to the inhibition of these kinases. Expression of the VDR target gene *CYP24A1* was not enhanced by resveratrol alone or in combination with  $1\alpha,25(\text{OH})_2\text{D}_3$  suggesting that the effect on *CAMP* expression was not due to an enhancement of vitamin D-signaling in general. The differential recruitment of transcriptional factors or cofactors to the *CAMP* gene promoter remains to be determined.

Resveratrol has been shown to induce endoplasmic reticulum stress and we have observed increased XBP-1 splicing in our cells treated with resveratrol (data not shown) [36, 37]. Furthermore, Park and colleagues showed that endoplasmic reticulum stress induced with either thapsigargin (Tg) or tunicamycin increased expression of the *CAMP* gene in HaCaT and normal human keratinocytes [38]. Nevertheless, they demonstrated that the induction of endoplasmic reticulum stress in the presence of  $1\alpha,25(\text{OH})_2\text{D}_3$  did not show a synergistic effect, but instead suppressed vitamin D-induced *CAMP* expression [38]. These findings would indicate that endoplasmic reticulum stress induced by resveratrol does not contribute to the synergy that we observed in this study.

Although, the mechanism by which resveratrol induces *CAMP* gene expression remains unclear, the discovery that resveratrol in combination with vitamin D enhances *CAMP* gene expression is intriguing and consistent with previous findings that a number of natural small molecules regulate *CAMP* expression [2]. The potential of combining vitamin D with stilbenoids to improve immunity remains to be determined. Bioavailability of stilbenoids upon their oral consumption is a problem as they are metabolized into glucuronated and sulfonated byproducts by the intestine and liver [39]. Nevertheless, topical applications to improve barrier defense in wounds or infections could be envisioned as active forms of vitamin D are used to treat psoriasis and resveratrol is used in cosmetics [40–42]. Interestingly, topical resveratrol inhibits herpes simplex virus replication *in vitro* and *in vivo* in mice [43, 44]. Future work is required to determine if vitamin D alone or in combination with resveratrol will be useful for boosting the innate immune response or barrier defense against infection.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

<b>(1<math>\alpha</math>,25 (OH)<sub>2</sub> D<sub>3</sub>)</b>	1 $\alpha$ ,25 hydroxyvitamin D <sub>3</sub>
<b>(CAMP)</b>	cathelicidin antimicrobial peptide
<b>(TSTA)</b>	Two Step Transcriptional Activator
<b>(VDR)</b>	Vitamin D Receptor

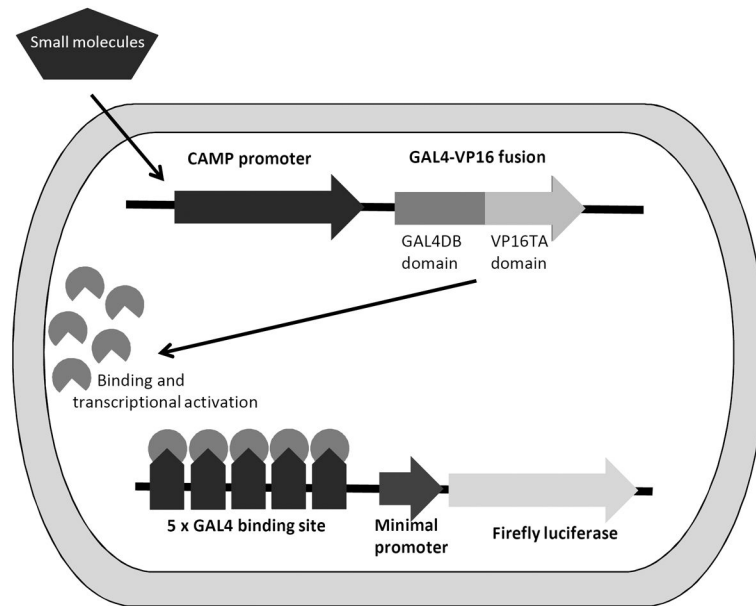
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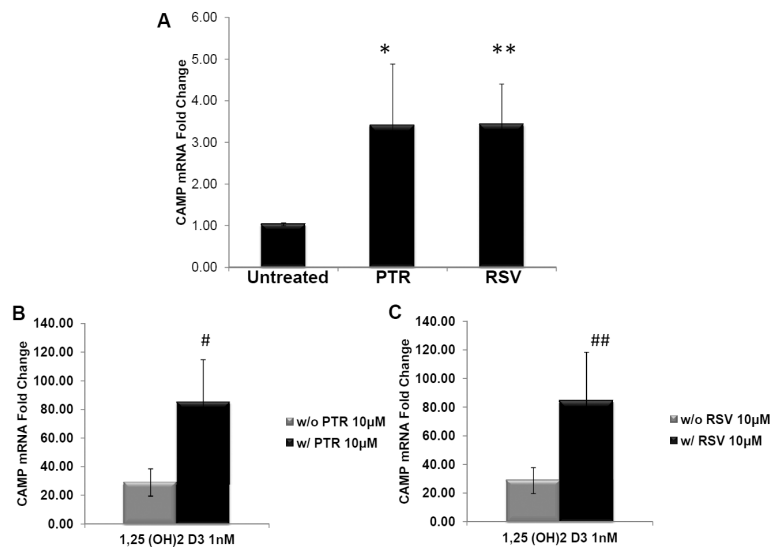
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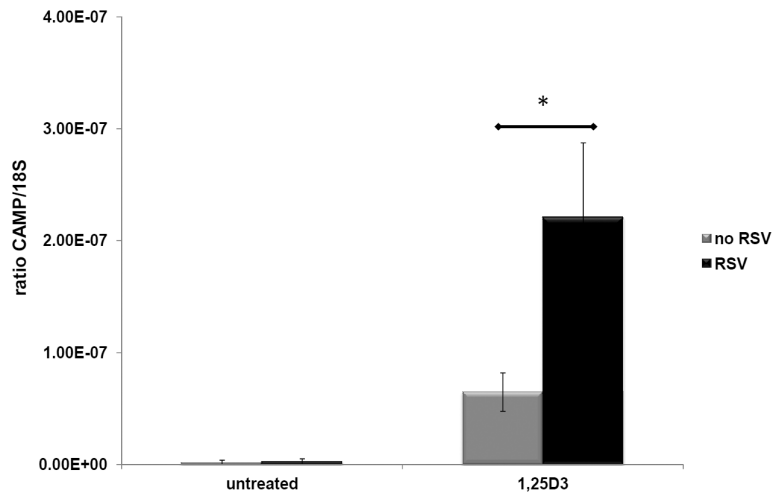


**Figure 1. Schematic of TSTA-hCAMP-FFL reporter plasmid**

Small molecules that induce expression from the human CAMP promoter lead to the expression of the GAL4-VP16 fusion transcription activator protein. This transcriptional activator binds to the five GAL4 binding sites upstream of the minimal promoter driving expression of the firefly luciferase (FFL) gene. Activation of the CAMP promoter by the small molecule is indirectly measured by the amount of luciferase activity [15].

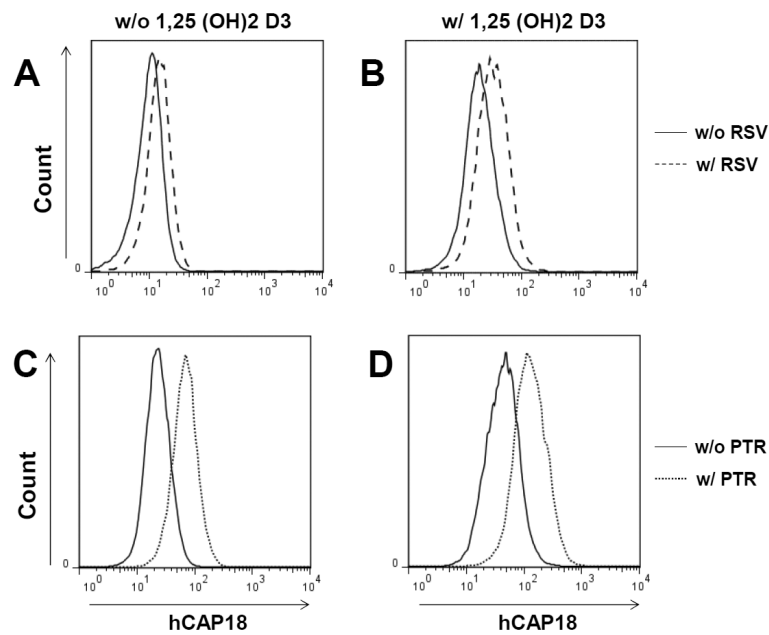


**Figure 2. Induction of endogenous CAMP gene expression by stilbenoid compounds** (Panel A) U937 cells were treated with either vehicle (untreated) 10  $\mu$ M pterostilbene (PTR) or resveratrol (RSV) for 18 h. Synergistic induction of CAMP gene expression by both stilbenoid compounds and 1,25(OH)<sub>2</sub>D<sub>3</sub>. U937 cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> and either without (w/out) or with (w/) 10  $\mu$ M PTR (panel B) or RSV (panel C). Levels of CAMP gene expression were measured by qRT-PCR and normalized to 18S rRNA levels. Results are shown as fold change compared to cells without the stilbenoid (panel A) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (panels B and C). Statistical significance was determined using a Student's t-test, \* $p=0.01$ ; \*\* $p<0.0001$ , # $p<0.05$  and ## $p<0.01$ .



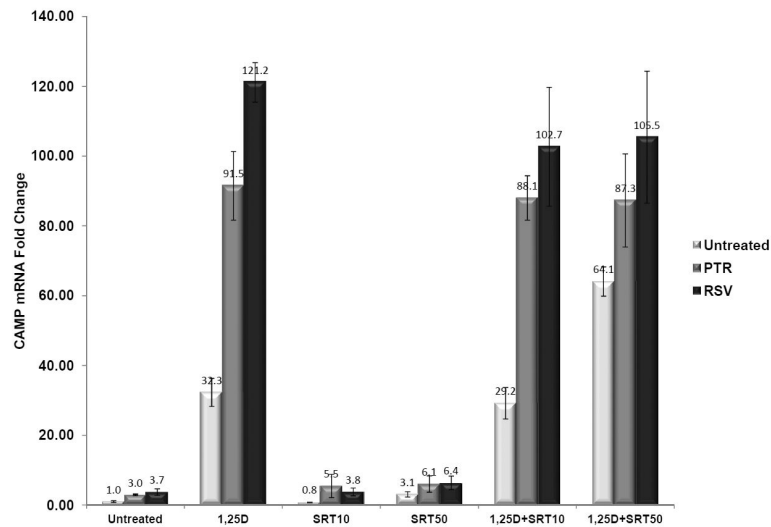
**Figure 3. Induction of endogenous CAMP gene expression by resveratrol (RSV) in combination with vehicle (untreated) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (1,25D<sub>3</sub>) in human keratinocytes**  
The human HaCaT cell line was treated with either ethanol vehicle, 10 μM RSV, 10 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or a combination for 18 h. Levels of CAMP gene expression were measured by qRT-PCR and normalized to 18S rRNA levels. Results are shown as a ratio of CAMP/18S. Statistical significance was determined using a Student's t-test, \*p=0.0007. Data are from two-independent experiments.





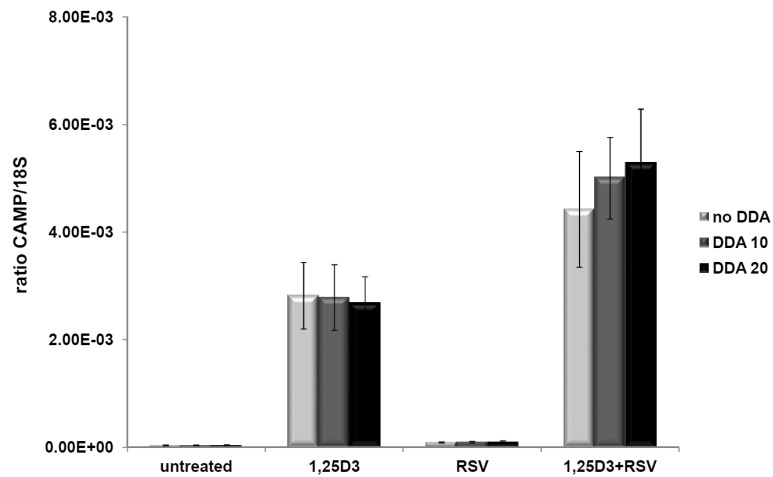
**Figure 4. Induction of cathelicidin protein (hCAP18) expression in U937 cells by stilbenoid compounds**

U937 cells were treated with either 10  $\mu$ M resveratrol (RSV, panel A) or 10  $\mu$ M pterostilbene (PTR, panel C) alone or in combination with 1 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (Panels B and D) for 24 h. Intracellular staining for hCAP18 and FACS was used to determine the expression level of hCAP18 in the cells. Results are representative of two independent experiments.



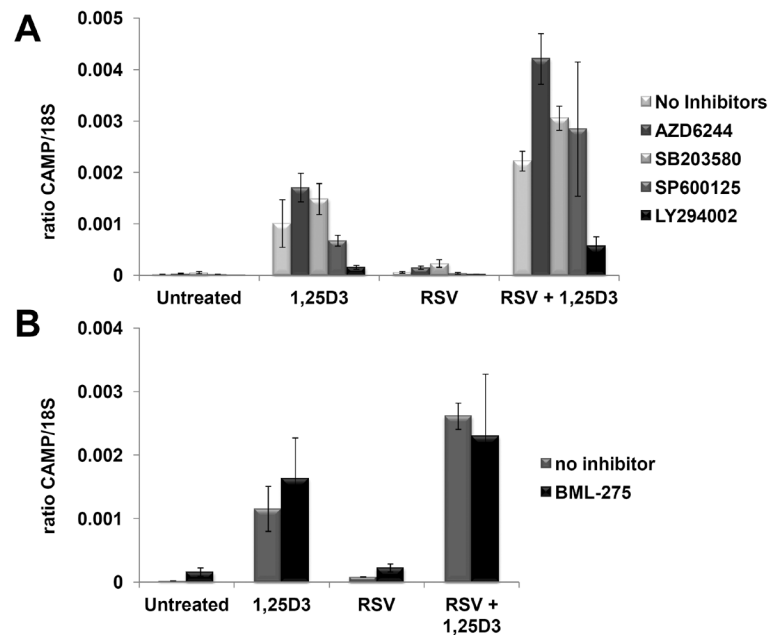
**Figure 5. Inhibition of SIRT1 does not block stilbenoid-mediated CAMP induction**

Effects of sirtinol on the synergistic induction of CAMP by 1,25(OH)<sub>2</sub>D<sub>3</sub> and either stilbenoid compound. U937 cells were treated with 10 μM of either pterostilbene (PTR) or resveratrol (RSV), and with or without 1nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (1,25D) or Sirtinol (10 or 50 μM, [SRT10 or SRT50], respectively) for 18 h. Sirtinol did not inhibit CAMP mRNA induction by either PTR or RSV. CAMP gene expression was determined by qRT-PCR and normalized to 18S rRNA levels. Changes in gene expression are represented as fold-change compared to the untreated control (first bar graph). Data presented are from one experiment, but representative of results from three individual experiments.



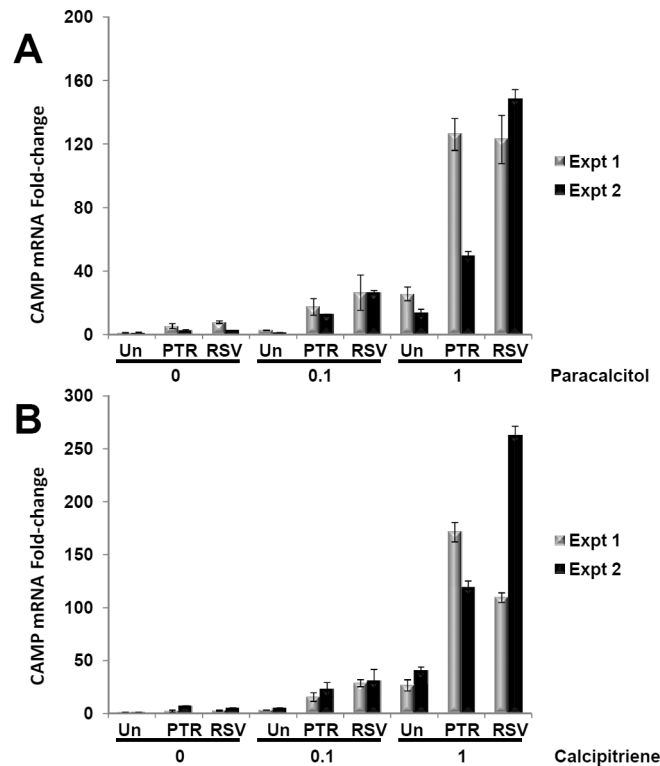
**Figure 6. 2',3'-dideoxyadenosine, a cAMP pathway inhibitor, did not affect resveratrol (RSV)-enhanced hCAMP expression**

U937 cells were treated with combinations of vehicle (untreated), 1,25(OH)<sub>2</sub>D<sub>3</sub> (1,25D3) or RSV in the presence of 2',3'-dideoxyadenosine (DDA) at 10 or 20 mM (DDA 10 or DDA 20, respectively) for 18 h. Levels of CAMP gene expression were measured by QRT-PCR and normalized to 18S rRNA levels. Results are shown as a ratio of CAMP/18S. The data represent two-independent experiments combined.



**Figure 7. Inhibition of the MAPK, PI3K and AMPK pathways does not block the effect of resveratrol (RSV) on CAMP gene expression**

(Panel A) U937 cells were treated with combinations of vehicle (untreated), 1,25(OH)<sub>2</sub>D<sub>3</sub> (1,25D3) or RSV in the presence or absence of inhibitors for ERK1/2 (AZD6244), p38 MAP kinase (SB203580), c-Jun kinase (SP600125) and PI3 kinase (LY294022) for 18 h. (Panel B) U937 were treated as described in panel A, but an inhibitor for AMP kinase, BML-275 was used. Levels of CAMP gene expression were measured by qRT-PCR and normalized to 18S rRNA levels. The data are from two individual experiments combined. Results are shown as a ratio of CAMP/18S.



**Figure 8. Synergistic induction of CAMP gene expression by stilbenoids and vitamin D analogs used in the clinic**

U937 cells were treated with vehicle (0) or vitamin D analog (0.1 or 1 nM paracalcitol or calcipitriene) in absence (Un) or presence of pterostilbene (PTR) or resveratrol (RSV) for 18 h. Levels of CAMP gene expression were measured by QRT-PCR and normalized to 18S rRNA levels. Changes in gene expression are represented as fold-change compared to the untreated control (no analog or stilbenoid). Data from two individual experiments are shown.