Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D₃


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Steroid hormones are central regulators of a variety of biological processes. According to the free hormone hypothesis, steroids enter target cells by passive diffusion. However, recently we demonstrated that 25(OH) vitamin D₃ complexed to its plasma carrier, the vitamin D-binding protein, enters renal proximal tubules by receptor-mediated endocytosis. Knockout mice lacking the endocytic receptor megalin lose 25(OH) vitamin D₃ in the urine and develop bone disease. Here, we report that cubilin, a membrane-associated protein colocalizing with megalin, facilitates the endocytic process by sequestering steroid–carrier complexes on the cellular surface before megalin-mediated internalization of the cubilin-bound ligand. Dogs with an inherited disorder affecting cubilin biosynthesis exhibit abnormal vitamin D metabolism. Similarly, human patients with mutations causing cubilin dysfunction exhibit urinary excretion of 25(OH) vitamin D₃. This observation identifies spontaneous mutations in an endocytic receptor pathway affecting cellular uptake and metabolism of a steroid hormone.

Here, we identify cubilin as an important coreceptor in the endocytic pathway for retrieval of 25(OH)D₃–DBP complexes by megalin-mediated endocytosis in the kidney. We show that absence of cubilin or inhibition of its function markedly reduces cellular uptake of the steroid–carrier complex, and animals or patients lacking functional cubilin are characterized by abnormal vitamin D metabolism. This study identifies patients with mutations in an endocytic pathway that regulates steroid hormone metabolism.

Materials and Methods

Ligands, Receptors, and Antibodies. DBP was purified from human serum (2). Receptor-associated protein (RAP) was produced in Escherichia coli (9); 3H-25(OH)D₃ was from Amersham Pharmaccia, and 25(OH)D₃ was from Dr. A.-M. Kissmeyer (Leo Pharmaceutical Products, Ballerup, Denmark). Biotin-25(OH)D₃ was synthesized by coupling 25(OH)D₃-3-(3′-aminopropyl)ether (10) with aminocaproic acid-biotin-4-nitrophenyl ester (Pierce) (11). Sterol–carrier complexes were prepared by incubating DBP with 10 to 100-fold excess labeled or unlabeled 25(OH)D₃ (2). Uncomplexed steroid was removed by gel filtration or dialysis. Human retinol-binding protein (RBP) was from Dr. G. Alexander (University of Oslo, Norway). Rabbit megalin and cubilin were purified as reported (5).

The primary antibodies used were rabbit anti-human DBP and anti-human RBP (Dako), goat anti-human DBP (DiaSorin, Stillwater, MN), rabbit anti-rat cubilin (12), and sheep anti-rat megalin (13). Primary antibodies were visualized by using rhodamine-labeled donkey anti-goat IgG (Abcam, Cambridge, U.K.), Cy5-coupled donkey anti-sheep antibody (Amersham Pharmacia), FITC-, tetramethylrhodamine B isothiocyanate-, and horseradish peroxidase-labeled swine anti-rabbit IgGs (Dako), and Alexa488 and Alexa546 conjugated donkey anti-sheep and goat anti-rabbit antibodies (Molecular Probes). Biotin-25(OH)D₃ was detected by using Cy5-coupled streptavidin (Amersham Pharmacia).

DBP Affinity Chromatography and Western Blotting. A DBP affinity column was generated by immobilizing 5 mg of purified human DBP on cyanogen bromide-activated Sepharose-4B. Chromatography was accomplished by incubating rabbit kidney cortex membranes (6) on the DBP column for 16 h at 4°C, followed by washing with 150 bed volumes of 10 mM Hepes/140 mM NaCl/2 mM CaCl₂/1 mM MgCl₂/0.6% CHAPS, pH 7.5. Bound proteins were eluted at pH 4.0 with 5 mM EDTA, 1-ml fractions were analyzed by gel filtration and Western blotting.

Abbreviations: DBP, vitamin D-binding protein; 25(OH)D₃, 25(OH) vitamin D₃; IF–B₁₂, intrinsic factor–vitamin B₁₂ complex; RAP, receptor-associated protein; RBP, retinol-binding protein; SPR, surface plasmon resonance.

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by 4–16% SDS/PAGE, and Western blotting was visualized by using enhanced chemiluminescence.

**Surface Plasmon Resonance (SPR) Analysis.** Receptor–ligand interactions were assessed by SPR analysis on a BIAcore 2000 (Biosensor) (2). The signal from immobilized proteins corresponded to 51 fmol of immobilized megalin/mm^2 and 42 fmol of immobilized cubilin/mm^2. The signal is expressed in relative response units (RU), i.e., the difference in response between protein and control flow channels. The data were evaluated by using the BIAEVALUATION 3.1 software.

**Cell Uptake Studies.** BN/MSW cells (14) and human keratinocytes (15) have been described. The cells were grown to confluence in 24-well plates and incubated with ~50 pM of labeled 25(OH)D_3–DBP in serum-free MEM containing 0.1% ovalbumin. As cell-associated _125^I_ DBP was negligible, degraded DBP (trichloroacetic acid-soluble radioactivity) was taken as a measure of cellular uptake. Uptake of the steroid was measured as cell-associated radioactivity after washing twice with MEM.

**Animal Models.** Mixed breed dogs exhibiting autosomal recessive inheritance of cubilin malexpression have been reported (16, 17). Megalin-deficient mice were produced by gene targeting as described (18).

**Urinary and Blood Samples.** Urine from Imerslund–Gräsbeck patients and control individuals was collected and frozen immediately (8). Urine and EDTA plasma were collected from mixed-bred cubilin malexpressing dogs and normal dogs of various strain, age, and sex. For the mice, urine was collected in metabolic cages (19), and EDTA plasma was obtained by retroorbital bleeding. Urine and blood parameters were measured by Nova Medical Medi-Lab (Copenhagen, Denmark) and by Animal Health Diagnostic Laboratory (East Lansing, MI) and analyzed by Student’s _t_ test unless otherwise indicated. Urinary excretion of 25(OH)D_3 was measured on samples concentrated 20-fold. The displayed values have been corrected accordingly.

**Immunostaining of Kidneys and Cells.** Dog and mouse kidneys were fixed with paraformaldehyde (19, 20). Semithin cryosections (6) were incubated on glass slides with primary antibody, followed by incubation for 1 h with secondary antibodies.

**Results**

**Cubilin Is a Receptor for DBP.** Possible coreceptors potentially important for uptake of 25(OH)D_3–DBP complexes in kidney tubules were identified by affinity chromatography using immobilized DBP and solubilized rabbit kidney membranes. Two proteins eluted from the DBP column, but not from a control column (Fig. 1a). The ~600-kDa protein corresponded to megalin in agreement with our previous observation that this receptor mediates uptake of 25(OH)D_3–DBP complexes in the kidney (2). The ~460-kDa protein was identified as the IF-B12 receptor cubilin, thus raising the possibility that cubilin binds DBP. However, as coelution could be due to the previously described interaction between the two receptors (7), we performed experiments using SPR analysis to demonstrate direct binding of DBP to purified cubilin. The results demonstrated Ca^{2+}-dependent binding of DBP to cubilin and confirmed its binding to megalin (Fig. 1b). The affinities for DBP binding were similar, with _K_d_ values estimated from five experiments at 110 ± 15 nM for cubilin and 120 ± 27 nM for megalin. The formation of complex with 25(OH)D_3 did not influence binding of DBP to either receptor (not shown).

To discriminate between binding of the steroid–carrier complex to the two receptors, we analyzed the effect of RAP, a 40-kDa endoplasmic reticulum-resident chaperone for megalin, other receptors of the low density lipoprotein receptor family (21), and

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**Fig. 1.** Characterization of DBP binding to cubilin and megalin. (a) DBP affinity chromatography of rabbit kidney membranes. Fractions eluted at pH 4.0 in the presence of 5 mM EDTA from the DBP affinity column (Left) or mock column (Right) were subjected to SDS/PAGE and silver staining. The two proteins eluted were identified as megalin (~600 kDa) and cubilin (~450 kDa) using Western blot analysis of fraction 11 (Center). (b–d) SPR analysis of DBP (1 μM) binding to immobilized megalin and cubilin. (b) The on and off rates were recorded, and the _K_d_ values were 133 nM and 129 nM for binding to megalin and cubilin in the displayed experiment, respectively. EDTA (10 mM) inhibits binding to both receptors. (c) RAP (10 μM) was prebound to immobilized megalin at 200–700 s followed by the addition of 0.5 μM DBP (full line) or continuous infusion with 10 μM RAP alone (dashed line). Binding of DBP after preincubation with buffer alone is shown for comparison. (d) RAP (10 μM) was prebound to cubilin at 200–700 s, and the experiment was carried out as described for megalin in c.
25(OH)D3 by up to 70%, whereas control IgG had no effect. Analysis of the DBP–25(OH)D3 moiety, allowing us to compare cellular uptake of each 25(OH)D3 with DBP binding to cubilin. This result allowed us to differentiate and RAP to different sites. Thus, RAP blocks megalin function and internalization of 25(OH)D3–DBP complexes by BN/MSV cells. We next used confocal fluorescence microscopy to visualize cooperation between cubilin and megalin in the uptake process, we measured internalization of 25(OH)D3–DBP complexes in cells. We first compared uptake of steroid–carrier complexes in BN/MSV cells that express megalin and cubilin in abundance, with uptake in keratinocytes that lack both receptors (Fig. 2a). The complexes were labeled in either the DBP or the 25(OH)D3 moiety, allowing us to compare cellular uptake of each component (Fig. 2b). After 2 h at 37°C, the BN/MSV cells had internalized more than 30% of the DBP and 25(OH)D3. By contrast, no major uptake was observed in keratinocytes, indicating that passive diffusion of the steroid through the plasma membrane was quantitatively insignificant. To investigate the relative contribution of cubilin and megalin in the uptake process, we measured internalization of 25(OH)D3–DBP complexes by BN/MSV cells in the presence of antibodies specific to each of the two receptors (Fig. 2c). Addition of anti-cubilin inhibited cellular uptake of DBP and 25(OH)D3 by up to 70%, whereas control IgG had no effect. Anti-megalin antibodies similarly reduced endocytic uptake by ~70%, thus confirming the importance of megalin for endocytosis of 25(OH)D3–DBP (2). The combined application of the two IgGs impaired uptake of DBP and 25(OH)D3 only slightly more (~80%) than each antibody alone, suggesting that cubilin and megalin function in the same endocytic pathway. As an internal control, uptake of RBP, the plasma carrier for retinol and an established ligand for megalin (22), was not affected by the anti-cubilin antibody, whereas IgG to megalin inhibited endocytosis of RBP by ~88% (data not shown).

The lack of a transmembrane domain suggests that cubilin needs assistance from megalin to perform endocytosis of its ligand. We therefore applied RAP to inhibit the interaction between cubilin and megalin and to block binding of 25(OH)D3–DBP directly to megalin (Fig. 2c). RAP virtually abolished internalization of the steroid–carrier complex, demonstrating that cubilin activity is not by itself sufficient to target the ligand complex to the interior of the cells. We next used confocal fluorescence microscopy to visualize the internalization process (Fig. 2d). Following 30 min of incubation, DBP was present in the endosomal compartment, whereas 25(OH)D3 appeared in the cytosol. After 2 h at 37°C, a strong perinuclear and lysosomal-like staining for DBP paralleled a considerable cytoplasmic accumulation of the steroid. However, in the presence of RAP, the cellular accumulation of DBP and 25(OH)D3 was completely inhibited. Thus, the data obtained in BN/MSV cells support a model in which cubilin is the principal binding site on the plasma membrane, followed by association between cubilin and megalin to achieve endocytic uptake of the cubilin-bound 25(OH)D3–DBP complex.

Cubilin Deficiency in Dogs Causes Disturbances in Vitamin D Metabolism. An unknown autosomal recessive defect causing selective malabsorption of IF–B12 and proteinuria, due to failure of apical membrane expression of cubilin in intestine and proximal tubules,
has been demonstrated in a family of giant schnauzer dogs (16). Similar to patients with Imerslund–Gräsbeck disease, these dogs exhibit severe vitamin B₁₂ deficiency, unless treated with parenteral vitamin B₁₂. We investigated the role of cubilin for endocytic retrieval of filtered 25(OH)D₃–DBP complexes in dog kidney. All samples from affected dogs were taken during complete hematopoietic remission caused by regular vitamin B₁₂ administration. Immunohistology of renal cortical cryosections (Fig. 3a) demonstrated that in the affected dogs, in contrast to control dogs, cubilin (green) did not colocalize with megalin (blue) on the apical surface of the proximal tubular epithelium, but was dispersed in vesicles throughout the cytosol and therefore not accessible to ligand in the tubular lumen. In control dogs, DBP antiserum showed a distinct labeling (red) of the endosomal compartment underneath the apical surface of the proximal tubules, indicating uptake of DBP from the glomerular filtrate. In affected dogs, DBP was also present in endocytic vesicles but at a reduced level, indicating that absence of cubilin on the cell surface impaired, but did not entirely abrogate, endocytosis of DBP. For comparison, tubules from megalin knockout mice showed no staining for DBP despite intact expression of cubilin, thus validating the vital role of megalin for the internalization process.

To confirm the reduced uptake of DBP in affected dogs, we collected urine samples and measured excretion of DBP and 25(OH)D₃ (Fig. 3b and Table 1). Affected dog urine contained significant amounts of DBP, whereas urine from control dogs did not. Urinary excretion was not due to a secondary effect on megalin activity because RBP, which binds to megalin only, did not accumulate in the urine. For comparison, urine from megalin-deficient mice contained both DBP and RBP. Notably, urinary loss of DBP in five cubilin-deficient animals was accompanied by significant urinary 25(OH)D₃ excretion with concentrations of 0.54 ± 0.19 nM as compared with nondetectable levels in seven control samples, demonstrating that cubilin partakes in the retrieval of filtered steroid (Table 1). In megalin knockout and wild-type mice, the corresponding values were 0.87 ± 0.01 nM and nondetectable, respectively. As expected, the megalin-deficient mice exhibited significant urinary excretion of retinol (0.25 ± 0.09 μM, P < 0.01, n = 4), whereas no excretion was detectable in the dogs. Finally, absence of high molecular weight proteinuria (not shown) and normal urinary creatinine concentrations in the receptor-deficient dogs and mice indicated that aberrant glomerular filtration did not account for the urinary excretion of 25(OH)D₃.

To evaluate the physiological significance of reduced 25(OH)D₃ reabsorption for vitamin D homeostasis, blood samples from the same animals were analyzed for vitamin D metabolites (Table 1). Urinary loss of 25(OH)D₃ in the cubilin-affected dogs was accompanied by a 45% reduction in plasma 25(OH)D₃ when compared with control animals (50.6 ± 16.6 nM versus 90.4 ± 30.6 nM, P < 0.05). More importantly, the bioactive metabolite 1,25(OH)₂D₃ was similarly decreased from 238.1 ± 91.8 pM in wild type to 137.0 ± 32.4 pM (P < 0.05) in affected dogs, underscoring the importance of cubilin in vitamin D homeostasis. For comparison, megalin deficiency, which completely disrupts reabsorption of the filtered steroid, caused a ∼70% reduction in plasma levels of both the mono- and dihydroxylated vitamin D₃ metabolites. To confirm the effect of cubilin malexpression on vitamin D₃ metabolism, we subsequently analyzed blood samples from a larger number of dogs. When eight cubilin-deficient animals were compared with 11 controls, the difference in 1,25(OH)₂D₃ levels was even more profound, being 211.1 ± 68.1 pM in the affected dogs versus 503.5 ± 154.7 pM in the controls (P < 0.001), corresponding to a ∼59% reduction in plasma 1,25(OH)₂D₃. Hence, lack of cubilin

### Table 1. Urinary excretion of 25(OH)D₃ and plasma vitamin D₃ metabolites in cubilin-diseased and megalin-deficient animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Affected dogs (n = 5)</th>
<th>Control dogs (n = 7)</th>
<th>P value</th>
<th>–/- mice (n = 4)</th>
<th>+/+ mice (n = 5)</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Cubilin</strong></td>
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<tr>
<td>Urine 25(OH)D₃</td>
<td>nmol/liter</td>
<td>0.54 ± 0.19</td>
<td>nd</td>
<td>&lt;0.01*</td>
<td>0.87 ± 0.01</td>
<td>nd</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mmol/liter</td>
<td>16.33 ± 7.73</td>
<td>16.67 ± 9.02</td>
<td>P = 0.95</td>
<td>1.80 ± 1.05</td>
<td>1.77 ± 1.02</td>
<td>P = 0.96</td>
</tr>
<tr>
<td>Plasma 25(OH)D₃</td>
<td>nmol/liter</td>
<td>50.60 ± 16.61</td>
<td>90.40 ± 30.60</td>
<td>P &lt; 0.05</td>
<td>27.50 ± 8.32</td>
<td>86.61 ± 20.40</td>
<td>P &lt; 0.01</td>
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<tr>
<td>1,25(OH)₂D₃</td>
<td>pmol/liter</td>
<td>137.01 ± 32.41</td>
<td>238.14 ± 91.80</td>
<td>P &lt; 0.05</td>
<td>43.31 ± 17.51</td>
<td>130.04 ± 42.31</td>
<td>P &lt; 0.01</td>
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<td><strong>Megalin</strong></td>
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<tr>
<td>Urine 25(OH)D₃</td>
<td>nmol/liter</td>
<td>0.87 ± 0.01</td>
<td>nd</td>
<td>&lt;0.01*</td>
<td>0.87 ± 0.01</td>
<td>nd</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mmol/liter</td>
<td>1.80 ± 1.05</td>
<td>1.77 ± 1.02</td>
<td>P = 0.96</td>
<td>1.80 ± 1.05</td>
<td>1.77 ± 1.02</td>
<td>P = 0.96</td>
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*nd indicates values below limit of detection; *<0.15 nM for 25(OH)D₃.

*Statistical analysis by the Mann-Whitney test.
25(OH)D3 is seen in patient 1 homozygous for the FM2 mutation, and retinol are shown. Limits of detection were 0.15 nM and 0.1 µM, respectively. The type of mutation present in the cubilin gene is shown. Creatinine is indicated for each patient.

Patients with Mutations in the Cubilin Gene Exhibit Urinary Loss of DBP and 25(OH)D3. Finally, we collected urine from patients with Imerslund–Gräsbeck disease to confirm the validity of the dog model in humans. Cubilin consists of a cluster of 27 CUB domains preceded by eight epidermal growth factor repeats and a putative amphipathic helix. Two mutations in the cubilin gene (FM1 and FM2, respectively) were recently identified in the Finnish population. The activation of the cryptic splice site, leading to an insertion in CUB domain 6, and an in-frame integration of multiple stop codons (8). As a consequence, patients carrying this mutation are expected to reabsorb DBP normally suggests that the binding site for IF–B12 is not known, but their mutations obviously also affect binding of DBP to the receptor demonstrated by urinary loss of 25(OH)D3 and DBP. Because no other mutations affecting IF–B12 metabolism have been disclosed in the cubilin gene, these patients may exhibit a defect in one or more unknown genes required for proper processing and sorting of cubilin (8), as was demonstrated in the cubilin-deficient dogs (17). None of the six patients exhibited urinary excretion of RBP and retinol, indicating intact megalin function. Furthermore, glomerular filtration was not different between patients and control individuals as determined by absence of high molecular weight proteinuria (data not shown) and comparable urinary excretion of creatinine.

Discussion
Steroid hormones regulate important biological processes including reproduction, metabolism, and skeletal formation. Surprisingly, the mechanism providing delivery of these hydrophobic molecules into target cells remains unclear. Although some uptake may be accounted for by diffusion of the free steroid (1), this process lacks specificity and is restricted by the small fraction of uncomplexed steroid in the circulation. Endocytic receptor pathways may therefore have evolved to target steroid–carrier complexes to cells with large requirements, e.g., for metabolic conversion of the steroids. The present study demonstrates that cubilin and megalin constitute a functional unit for delivery of 25(OH)D3 to renal proximal tubule cells.

Cubilin was first identified as a receptor for IF–B12 complex in the terminal ileum (6). As direct association between cubilin and megalin has been demonstrated (7), it was suggested that molecular cooperation provides the basis for internalization of ligands bound to cubilin. Thus, cubilin with bound ligand may undergo megalin-mediated endocytosis, unload its cargo in lysosomes, and recycle back to the plasma membrane together with megalin (7, 23).

Our data show that although 25(OH)D3–DBP can bind directly to megalin, cubilin greatly facilitates the endocytic process by sequestering the steroid–carrier complex on the cell surface before internalization via megalin. This pathway rescues steroid from urinary excretion and ensures sufficient substrate for generation of 1,25(OH)2D3 by the 1α-hydroxylase (Fig. 5).
The importance of cubilin in the endocytic process is underscored by the 70% reduction in 25(OH)D3–DBP uptake in BN/MSV cells following inhibition of cubilin function. The physiological importance is demonstrated by the consistent differences in plasma vitamin D3 parameters between controls and cubilin-deficient dogs, even though animals of both groups were housed and fed differently and were not genetically defined except with regard to the cubilin malexpression locus. However, no statistically significant difference in PTH levels was found between cubilin-affected and control dogs (4.8 ± 1.8 pM versus 3.5 ± 0.9 pM, respectively, P = 0.14, n = 10), indicating that the decrease in plasma 1,25(OH)2D3 levels did not cause secondary hyperparathyroidism. However, all dogs were on high quality diets, and it is possible that changes in PTH levels due to cubilin malfunction may only be observed when dietary vitamin D intake is suboptimal. It is evident that environmental factors interact with genetic determinants of nutrient utilization to produce normal or abnormal states of health. For instance, mutations in the sodium/iode symporter expressed in thyroid follicular cells that do not cause hypothyroidism when there is sufficient dietary iodine may become clinically apparent only when dietary iodine intake is suboptimal (24).

In the cubilin-deficient dogs, plasma 1,25(OH)2D3 was reduced ~59% as compared with about 70% in megalin-deficient mice. Although cubilin has previously been identified as a receptor for albumin, apolipoprotein A1, and Clara cell secretory protein in the kidney (19, 20, 25), the overall consequences of cubilin deficiency in metabolism of these ligands remain unclear. To our knowledge, cubilin has previously been identified as a receptor for 1α-hydroxylase, which is responsible for converting 25(OH)D3 to calcitriol, at least when production in the proximal tubules fails, as in the megalin- and cubilin-deficient animals. A detailed analysis of the vitamin D homeostasis in Immunslund–Grässbeck disease, as compared with suitable controls, was preceded by the limited number of patients and the fact that plasma calcitriol levels vary with diet and seasons. However, patients exhibiting urinary loss of DBP and 25(OH)D3 are likely to experience reduced plasma 1,25(OH)2D3 levels, as did the cubilin-deficient dogs. Interestingly, nonspecific rheumatic symptoms like diffuse bone pain, muscle ache, and fatigue may develop as the result of even moderate reductions (~40%) in plasma 25(OH)D3 and 1,25(OH)2D3 levels without biochemical or clinical signs of osteopathy (27). Such symptoms may easily be overlooked or considered a result of the anemia in Immunslund–Grässbeck patients. Future studies of hypovitaminosis D patients may reveal other mutations in the cubilin gene that selectively affect binding and uptake of 25(OH)D3–DBP.

In conclusion, cubilin is a physiologically important coreceptor for megalin-mediated delivery of the steroid 25(OH)D3 to kidney epithelial cells, and we identify mutations in an endocytic pathway leading to abnormal steroid metabolism. Similar endocytic pathways may exist for other steroid hormones, and identification of such pathways may have important clinical and pharmaceutical implications.

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