1,25-Dihydroxyvitamin D₃ Stimulates Expression and Translocation of Protein Kinase Cα and Cδ via a Nongenomic Mechanism and Rapidly Induces Phosphorylation of a 33-kDa Protein in Acute Promyelocytic NB4 Cells*

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NB4 cells are the only in vitro model for the study of acute promyelocytic leukemia (1). These cells contain the characteristic translocation (15;17) that disrupts the retinoic acid receptor and is thought to be the major contributor to the leukemic phenotype in acute promyelocytic leukemia patients (2). The majority of acute promyelocytic leukemia blasts from patients and the NB4 cell line in culture respond to pharmacologic doses of all-trans-retinoic acid by differentiating along the neutrophilic pathway. Remissions are frequent in diseased patients; however, relapse usually ensues as a result of the development of retinoic acid resistance (3). The mechanisms responsible for resistance are likely to include increased expression of cellular retinoic acid-binding proteins. This has prompted investigators, including ourselves, to consider the possibility of initiating cellular differentiation in the monocytic lineage as a potential treatment to be used cooperatively with all-trans-retinoic acid treatment or as an alternative therapy should all-trans-retinoic acid fail.

We recently succeeded in inducing monocytic differentiation of NB4 cells using combinations of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) (4) and TPA (4). We also demonstrated that the nongenomic analogue 1α,25-dihydroxyvitamin D₃ (HF) increased expression of PKCα and PKCδ. PKCα and PKCδ were translocated to the nucleus of the cell in response to 1,25-(OH)₂D₃ or HF. The effects of HF were attenuated by the nongenomic antagonist 1β,25-dihydroxyvitamin D₃, suggesting that changes in PKC expression are mediated by a nongenomic signaling pathway. Consistent with the involvement of serine, threonine, and tyrosine phosphorylation cascades mediating 1,25-(OH)₂D₃ action, enhanced phosphorylation of a variety of cellular proteins at serine and threonine residues and the specific enhanced phosphotyrosyl content of a 33-kDa protein (vdrp33) were observed immediately after 1,25-(OH)₂D₃ addition. We propose that 1,25-(OH)₂D₃ primes NB4 cells for 12-O-tetradecanoylphorbol-13-acetate-induced monocytic differentiation by increasing the expression of specific PKC isoforms and inducing the specific phosphorylation of key protein signaling intermediates.

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1 The abbreviations used are: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; TPA, 12-O-tetradecanoylphorbol-13-acetate; HF, 1α,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; PKC, protein kinase C; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; Ab, antibody.
MONOCYTIC DIFFERENTIATION

We also identify an early response protein phosphorylated on tyrosine that we believe may represent a key signaling intermediate.

MATERIALS AND METHODS

Cell Culture—NB4 cells were grown in liquid suspension culture at densities between 2.0 and 8.0 × 10^6 cells/ml. Cells were cultured in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum and 50 units/ml penicillin and streptomycin from passages 5 to 42, after which time cells were unresponsive to growth factors and chemical agents and became senescent. Cultures were maintained in 5% CO2 in air at a modifiedified atmosphere at 37°C. Cell counts were routinely determined using a Coulter Counter (Model ZM), and cell viability was determined by trypan blue dye exclusion. Cell viability was >85% in all experiments. For differentiation experiments, cells were plated at initial densities of 2.0 × 10^6 cells/ml with 2.0 × 10^-7 M 1,25-dihydroxyvitamin D3 and 2.0 × 10^-7 M phorbol ester (TPA) alone, in combination, or sequentially (1,25-(OH)2D3, and then TPA). HF and HL were generous gifts from Dr. Anthony Norman (Riverside, CA). They were prepared in ethanol to give a stock concentration of 10^3 M. All blots were subsequently stripped and reprobed following the procedure recommended by Amersham Corp. All antibodies were of the mouse IgG isotype (Sigma). Preparations of antibody binding to proteins on the PVDF membrane under these conditions, except for a major 45-kDa protein on the antiphosphoserine blots that maintained a high level of reactivity in the presence of 1 mM phosphoserine.

Subcellular Fractionation—Cells were grown as described above. 24 h after treatment, cells were pelleted at 500 × g and washed two times in PBS-A (plus protease and phosphatase inhibitors), and an aliquot was immediately lysed according to the standard protocol. The remaining cells were suspended in cold lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, and protease inhibitors) and then disrupted by Dounce homogenization, and fractions were separated by differential centrifugation. Cell breakage was confirmed microscopically and resulted in >98% cell lysis. The pellet of a 10-min centrifugation at 800 × g represented the nuclear fraction. The supernatant was centrifuged for 1 h at 30,000 × g to isolate the particulate fraction (pellet). Protein content of the fractions was determined by the method of Bradford (Bio-Rad). Fractions were suspended in Laemmli buffer, heated to 40°C, sonicated for 45 s, and frozen for subsequent SDS-polyacrylamide gel electrophoresis.

RESULTS

Our previous experiments have indicated that the PKC inhibitors staurosporine and GF 109023X attenuated the differentiative response to either 1,25-(OH)2D3 or TPA, suggesting that PKC may mediate some of the effects of these agents (8). Our first experiment set out to determine which PKC isoforms were present in NB4 cells. We used a panel of antibodies specific for PKCa, -β, -δ, -ε, and -θ. PKCa and PKCb blots were probed using primary antibody concentrations of 1:1000 and 1:1500, respectively. A primary Ab concentration of 1:250 was used for the β, ε, and θ isoforms. The secondary Ab concentration was 1:2,500 for all lanes. The PKCa, -β, and -δ lanes were loaded with 4.5 × 10^6 cells/lane, and the PKCa and PKCb lanes were loaded with 8 × 10^6 cells/lane. Exposure time was 1 h.

![Fig. 1. NB4 cells express PKCa and PKCb, but not PKCβ, -δ, -ε, or -θ. Control NB4 cells cultured at 2 × 10^6 cells/ml were harvested, run on an 10% SDS-polyacrylamide gel, transferred to PVDF membranes, and probed with antibodies specific for PKCa, -β, -δ, -ε, and -θ. PKCa and PKCb blots were probed using primary antibody concentrations of 1:1000 and 1:1500, respectively. A primary Ab concentration of 1:250 was used for the β, ε, and θ isoforms. The secondary Ab concentration was 1:2,500 for all lanes. The PKCa, -β, and -δ lanes were loaded with 4.5 × 10^6 cells/lane, and the PKCa and PKCb lanes were loaded with 8 × 10^6 cells/lane. Exposure time was 1 h.](image-url)
expression by 80%, and PKC\(d\) expression was reduced to undetectable levels (Fig. 3, B and E). There were no increases in either PKC isoform in response to HL alone (Fig. 3, C and F).

Changes in PKC translocation, particularly of the \(\alpha\) isoform, have been associated with activation of the enzyme (11). We therefore sought to determine whether 1,25-(OH)\(_2\)D\(_3\) had the ability to not only increase PKC expression, but also to induce changes in the subcellular localization of either isoform. Control NB4 cells (t = 0) or cells incubated for 24 h with vehicle, 1,25-(OH)\(_2\)D\(_3\), HF, HL, or HF and HL were either immediately extracted in Laemmli buffer or subjected to a fractionation procedure that yielded a particulate and a nuclear fraction. PKC\(a\) and PKC\(d\) content of each fraction was determined by SDS-polyacrylamide gel electrophoresis and Western blot analysis. In control NB4 cells, PKC\(a\) is presumably primarily cytosolic because it was undetectable in the particulate and nuclear fractions (Fig. 4A). After 24 h, ethanol alone had a small effect on PKC\(a\) distribution, leading to an apparent increase in the amount of PKC\(a\) in the nuclear fraction (Fig. 4B). Treatment with 200 nM 1,25-(OH)\(_2\)D\(_3\) or 200 nM HF led to an increase in nuclear PKC\(a\) that was 7-fold higher than was found in the nuclear fraction of vehicle-treated cells (Fig. 4, B and C). PKC\(a\) was also translocated to the particulate fraction of both 200 nM 1,25-(OH)\(_2\)D\(_3\) and 200 nM HF-treated cells to levels of similar magnitude (Fig. 4, B and C). In NB4 cells treated with 10 nM HF, there was an increase in PKC\(a\) content in the nuclear fraction that was also 7 times higher than in vehicle-treated cells. In contrast to the 200 nM HF group, there was no appearance of detectable PKC\(a\) in the particulate fractions of cells treated with 10 nM HF (Fig. 4B). This does not exclude the possibility that PKC\(a\) is translocated to the particulate fraction of this treatment group; it is possible that translocation occurs, but is not sustained up to the 24-h time point. Treatment with 200 nM HL, the antagonist, did not result in altered PKC\(a\)
1,25-(OH)₂D₃ Stimulates PKCa/δ Expression/Translocation

**Fig. 4. Translocation of PKCa and PKCd in response to 1,25-(OH)₂D₃ and nongenomic analogues.** NB4 cells were plated in culture dishes at a density of 2 × 10⁶ cells/ml and treated with ethanol (vehicle), 200 nM authentic 1,25-(OH)₂D₃, 10 or 200 nM HF, 200 nM HL, or 100 nM HF and 200 nM HL for 24 h. After treatment, cells were either lysed immediately or subjected to the fractionation procedure described under “Materials and Methods.” 18 μg of protein from each fraction (whole (W), nuclear (N), and particulate (P)) were run on 10% SDS-polyacrylamide gel and transferred to PVDF membranes. Blots were then probed with antibodies specific for PKCa (A–C) or PKCd (D–F). The primary Ab concentrations used were 1:1000 (PKCa) and 1:500 (PKCd), with a secondary Ab concentration of 1:25,000. The exposure time for all blots was 12 h. Blots were analyzed by densitometry and are expressed as arbitrary units under each lane. Results are representative of three separate experiments.

authentic 1,25-(OH)₂D₃ also led to nuclear translocation of PKCd, but in contrast to the analogues, there was no detectable PKCa in the particulate fractions at 24 h (Fig. 4E). It is possible that translocation took place earlier in the time course. It should be noted that the quantity of PKCd, as determined by densitometry, was 2-fold higher in the nuclear fraction of the 1,25-(OH)₂D₃ group compared with the HF groups. Treatment with 200 nM HL had no effect on PKCa translocation and antagonized the effects of HF, so PKCa expression was not detectable in the nuclear and particulate fractions (Fig. 4F).

Our previous experiments not only implicated PKC in the response of NB4 cells to 1,25-(OH)₂D₃ priming for monocytic differentiation, but also suggested that tyrosine kinase signaling cascades were involved. Thus, we next studied the phosphorylation profiles of NB4 cells at early and later time points after 1,25-(OH)₂D₃ addition in an attempt to identify possible targets of 1,25-(OH)₂D₃ activity. Using monoclonal antibodies to the three individual phosphoamino acids, we were able to independently examine changes in phosphoserine, phosphothreonine, or phosphotyrosine of proteins separated by SDS-polyacrylamide gel electrophoresis. Specificity of antibody binding was assessed by competition with free phosphoamino acids (see "Materials and Methods").

Within 1 h of 1,25-(OH)₂D₃ addition to NB4 cells, a substantial increase in total phosphoserine was observed in multiple species over the entire molecular mass range resolved on a 10% gel (Fig. 5A). Between 1 and 4 h, phosphoserine (as well as phosphothreonine and phosphotyrosine; see below) content decreased substantially to the point of being nearly undetectable in some experiments by Western blotting. The average decrease was to 14 ± 5% of maximum antibody binding (taken as 100%) as assessed by densitometry of the major phosphorylated species (n = eight independent experiments; p = 0.0023). Initially, we suspected that protein degradation had occurred in these samples, but fast green staining of replicate samples indicated that the lanes were equally loaded and expressed the entire range of protein molecular masses (Fig. 5D). By 12 h, the phosphoserine profile resembled that seen at 30 min to 1 h and thereafter slowly decayed. TPA alone induced a somewhat different profile of phosphoserine-containing proteins compared with 1,25-(OH)₂D₃. In particular, proteins of 30–40 kDa appeared to maintain their phosphoserine content in TPA-treated cells 24–48 h after the agent was added, while in 1,25-(OH)₂D₃-treated cells, phosphoserine gradually disappeared from proteins of this molecular mass range. On the whole, the phosphoserine profile of combination-treated cells was similar to that of untreated NB4 cells and had considerably less phosphoserine content than TPA only-treated cultures. The example shown is representative of at least four similar experiments.

Western blotting with antiphosphothreonine-specific antibody (Fig. 5B) revealed a similar pattern to that seen for antiphosphoserine, with a gradual increase in the phosphothreonine content of multiple species over the first hour of 1,25-(OH)₂D₃ treatment, a loss of phosphothreonine-containing proteins at 4 h, and rebound of the complete profile by 12 h, with subsequent decay back to the base line (untreated levels). Unique to the phosphothreonine blot, however, was what appeared to be the specific phosphorylation of a small molecular mass protein (~35 kDa) in TPA only-treated NB4 cells. This species was not observed in untreated, 1,25-(OH)₂D₃-treated, or combination (1,25-(OH)₂D₃/TPA)-treated NB4 cells.

The antiphosphotyrosine Western blotting revealed a phosphoprotein profile distinct from both the phosphoserine and phosphothreonine blots. Phosphotyrosine was rare on proteins from untreated NB4 cells (Fig. 5C). However, immediately
files, all detectable phosphotyrosine-containing species disappeared at 4 h, reappeared at 12 h, and decayed thereafter. TPA-treated cultures also demonstrated substantial phosphotyrosine content of many of the same molecular mass species observed in 1,25-(OH)_{2}D_{3}-treated cells. However, the unique difference in TPA-treated cells was that at 48 h, the 33-kDa band remained highly phosphorylated at tyrosine, while at this point in the 1,25-(OH)_{2}D_{3} treatment, the 33-kDa band was barely detectable. In combination-treated cells, the profile resembled that of 1,25-(OH)_{2}D_{3}-treated cells except that the relative intensity of the 33-kDa band to other higher molecular mass species was lower than that seen in 1,25-(OH)_{2}D_{3} only-treated cells. The 35-–40-kDa threonine-phosphorylated and tyrosine-phosphorylated species from TPA-treated cells appeared to comigrate, but it cannot be determined from these studies whether they represent dual phosphorylation of the same protein at both residues.

**DISCUSSION**

Previously, we have shown that 1,25-(OH)_{2}D_{3} primes NB4 cells for monocytic differentiation by a pathway that is independent of VDR binding (6). We also gave indirect evidence that PKC and tyrosine kinase signaling cascades were involved through the use of chemical inhibitors or activators of kinases and phosphatases (8). Here we have confirmed that 1,25-(OH)_{2}D_{3} indeed modulates expression of both PKC\(\alpha\) and PKC\(\delta\), the only two isoforms identified in these cells. Within a few hours of 1,25-(OH)_{2}D_{3} addition, there is increased expression of both PKC isoforms and translocation to the particulate (PKC\(\alpha\)) and nuclear (PKC\(\delta\)) compartments. This response appears to occur via nongenomic pathways since the 6-cis conformer (HF) was at least as efficient as authentic 1,25-(OH)_{2}D_{3} in up-regulating PKC expression and promoting translocation of both PKC\(\alpha\) and PKC\(\delta\) to the particulate and nuclear fractions of the cell. Furthermore, these activities were antagonized by the nongenomic antagonist HL.

Various PKC isoforms have been found to play essential roles in cellular differentiation processes. These include erythroid and monocytic differentiation of leukemia cells (13). Aihara et al. (14) suggested that sustained PKC activation was required for HL-60 differentiation into macrophages, and more recently, Gamard et al. (9) described a requirement for PKC\(\beta\) activity for HL-60 differentiation in response to 1,25-(OH)_{2}D_{3}. Macfarlane and Manzel (10) supported the pivotal role of PKC\(\beta\) in HL-60 monocytic differentiation by demonstrating that PKC\(\beta\) expression was sufficient for phorbol ester-induced differentiation. However, this conclusion must be challenged given that a recent study reported by Ryves et al. (15), comparing phorbol and 12-deoxyphorbol esters, suggested that PKC\(\beta\) activation was not sufficient to drive HL-60 cell differentiation. In addition, Mischak et al. (16) recently demonstrated that overexpression of PKC\(\alpha\) or PKC\(\delta\) resulted in TPA-induced monocytic differentiation of 32-D cells, while overexpression of PKC\(\beta\) (\(-\gamma\), \(-\epsilon\), \(-\zeta\), or \(-\eta\)) did not produce a differentiation response to TPA. NB4 cells do not express the \(\gamma\) or \(\zeta\) form of PKC, even in response to 1,25-(OH)_{2}D_{3}; thus, PKC\(\beta\) is clearly not responsible for the "priming" response to 1,25-(OH)_{2}D_{3}. However, because NB4 cells do not differentiate in response to 1,25-(OH)_{2}D_{3} in the absence of subsequent treatment with TPA, one could argue that the absence of PKC\(\beta\) prevents a full differentiation response to 1,25-(OH)_{2}D_{3} in this cell line. Because PKC\(\alpha\) and PKC\(\delta\) are coordinately regulated, we cannot determine whether both isoforms are necessary for the priming response to 1,25-(OH)_{2}D_{3} and the 6-cis analogue.

Both PKC\(\alpha\) and PKC\(\beta\) have been shown to translocate to the nucleus and may be modulated independent of each other by different stimuli (reviewed in Ref. 11). Binding proteins have
been implicated in PKC nuclear localization (17); however, there is some evidence that PKC may bind to membranes independent of binding proteins (18). In muscle cells, there is evidence that PKC may directly modulate the activity of DNA and RNA polymerases, histones, and transcription factors and thereby regulate the expression of muscle-specific genes (reviewed in Ref. 11). It is therefore conceivable that nuclear PKCα or PKCβ may directly phosphorylate and activate/inactivate transcription factors involved in the differentiation response of NB4 cells in response to 1,25-(OH)\(_2\)D\(_3\) and TPA. Other PKC substrates include p90 and p52, primarily on serine residues (19); the MARCKS protein (20); and the tissue-specific substrates neuromodulin (21) and neurogranin (22) in brain and p40–p47 in hematopoietic cells (23). Recently, it was shown that Raf-1 can be directly activated by PKC in murine hematopoietic cells, suggesting that PKC and growth factor signaling pathways may converge on the Raf-1 kinase (24). In the latter instance, it is not clear which isoforms were responsible for Raf-1 phosphorylation; however, Kolch et al. (25) identified Ser-499 of Raf-1 as the major phosphorylation site for PKCβ. Whether phosphorylation of these or the many other potential substrates of PKC plays a pivotal role in the differentiation process in NB4 cells or other cell types remains to be determined.

Consistent with a role for tyrosine, serine, and threonine kinase activity in the priming activity of 1,25-(OH)\(_2\)D\(_3\), substantial changes in the phosphoprotein profiles of NB4 cells were observed. Many species over the entire molecular mass range seemed to have increased content of phosphoproteins, peaking at 1 h, rapidly being dephosphorylated by 1 and 4 h, and then peaking again at 12 h, with a subsequent decline back to the base line. This biphasic kinetic pattern is unique to 1,25-(OH)\(_2\)D\(_3\) treatment since TPA treatment resulted in sustained phosphorylation beyond 48 h of treatment. In particular, the phosphorylation of a 40–45 kDa protein seems to stand out boldly in the TPA only-treated lysates. The combination treatment, on the other hand, more closely resembles the 1,25-(OH)\(_2\)D\(_3\) pattern, at least at the 48-h time point examined in this study. Whether this is coincidence or indicates a requirement for a specific phosphorylation profile to achieve a differentiation response needs to be examined. The phosphothreonine and phosphotyrosine profiles resembled the phosphoserine profile in terms of the time course of the response to 1,25-(OH)\(_2\)D\(_3\) and the patterns of phosphorylated proteins were similar between the three treatment groups (TPA, 1,25-(OH)\(_2\)D\(_3\), and 1,25-(OH)\(_2\)D\(_3\)/TPA). However, more obvious than in the phosphoserine blot, a protein of \(\sim 30–35\) kDa appears to be hyperphosphorylated at threonine in the TPA only-treated cultures, but is absent in the combination-treated cultures. The identity of this protein is currently unknown. A similar molecular mass protein, which we have tentatively named vdrp33 (33 kDa vitamin D response protein), is prominently tyrosine-phosphorylated within 1 min of 1,25-(OH)\(_2\)D\(_3\) addition and peaks first at 1 h, disappears, and then reappears at 12 h and decays back to the base line by 48 h. A protein of similar molecular mass is seen in both the combination-treated and TPA only-treated lysates, and once again, the TPA-only band appears to be hyperphosphorylated. In addition, a protein of \(-44\) kDa also appears to have sustained phosphorylation at tyrosine in TPA only-treated lysates. It is reasonable to speculate that the tyrosine-hyperphosphorylated species and the threonine-hyperphosphorylated species are in fact the same protein, but of course comigration does not prove this.

The considerable dephosphorylation of a variety of proteins that we observed to occur at 4 h and again closer to 48 h following 1,25-(OH)\(_2\)D\(_3\) addition may be the result of phosphatase activation. Dephosphorylation of the retinoblastoma gene has been demonstrated in human keratinocytes in response to 1,25-(OH)\(_2\)D\(_3\) (26) and is thought to be responsible for the G1/G0 growth arrest induced by 1,25-(OH)\(_2\)D\(_3\). Also, Omary et al. (27) recently reported the specific translocation of protein phosphatase 1 catalytic subunits during the monocytic differentiation of HL-60 cells in response to 1,25-(OH)\(_2\)D\(_3\). Although these authors demonstrated that inhibition of the phosphatase with calyculin A enhanced the 1,25-(OH)\(_2\)D\(_3\) differentiation response, it is certainly possible that redistribution and activation of the phosphatase are necessary for later differentiation events in NB4 cells. Thus, inactivation by translocation may be necessary for the initial events (i.e., the increased phosphorylation of a number of species), and dephosphorylation then becomes necessary to terminate the signal or to allow another signaling pathway to take over. Because 1,25-(OH)\(_2\)D\(_3\) treatment does not on its own result in a differentiation response, one might conclude that changes in phosphorylation of a specific set of proteins are necessary but not sufficient for monocyte development.

Although our PKC inhibitor studies documented that active PKC is necessary for monocytes differentiation of NB4 cells (8) in response to 1,25-(OH)\(_2\)D\(_3\) and TPA, the results of the present study suggest that PKC activation is not sufficient for differentiation induction. TPA alone also induced PKCα and PKCδ expression (this study), but this does not lead to cellular differentiation (8). Yada et al. (28) reported a similar “necessary but not sufficient” requirement for PKC activation in the differentiation of keratinocytes in response to 1,25-(OH)\(_2\)D\(_3\). Kindregan et al. (29) demonstrated a similar phenomenon in F9 teratocarcinoma cells. Yet another study showed that phorbol ester-resistant HL-60 cells were still capable of differentiating in response to 1,25-(OH)\(_2\)D\(_3\) (30). The signals that are unique to 1,25-(OH)\(_2\)D\(_3\) or the combination of 1,25-(OH)\(_2\)D\(_3\) and TPA that result in the fully differentiated phenotype have yet to be determined.

In addition to being a substrate for PKCβ (at serine 51), the VDR itself may also be a substrate for casein kinase II and other kinases (31). We have previously shown that analogues of 1α,25-(OH)\(_2\)D\(_3\), unable to bind the VDR, are even more potent inducers of differentiation than 1α,25-(OH)\(_2\)D\(_3\). However, this does not mean that VDR/VDR element responses are absent. Given that the VDR is a target for many kinases, it is conceivable that activation of VDR transcriptional activity may take place in the absence of ligand-receptor complexes. Our previous studies and this report strongly implicate PKC and tyrosine kinases as key mediators of the response to 1,25-(OH)\(_2\)D\(_3\). The molecular targets of these activities remain to be identified, and the relative importance of these novel pathways for 1,25-(OH)\(_2\)D\(_3\) action to the differentiation response of NB4 and other cell types needs to be examined. This is particularly important, given that the clinical utility of vitamin D analogues has usually been assumed to require VDR binding, a feature that usually also means that the analogues have high affinity for vitamin D-binding proteins that sequester the analogues out of the active compartment (32–34). Should activation of the differentiation program be achievable without VDR binding, this may provide an alternative strategy for differentiation therapy for diseases such as psoriasis, osteoporosis, and cancer. This of course assumes that the calcemic effects can be further dissociated from the differentiative effects by building a new generation of nongenomic 1,25-(OH)\(_2\)D\(_3\) analogues.

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