24-Oxo Metabolites of Vitamin D₃ Analogues: Disassociation of Their Prominent Antileukemic Effects from Their Lack of Calcium Modulation

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INTRODUCTION

The seco-steroid hormone, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] inhibits proliferation and induces differentiation of malignant cells including those of the hematopoietic system. The 24-oxo metabolite of 1,25(OH)₂D₃ also has prominent antiproliferative activities against various cancer cells. We chemically synthesized five novel 24-oxo vitamin D₃ analogues and evaluated their abilities both to inhibit clonal growth and induce differentiation of myeloid leukemia cells and to cause hypercalcemia. The 1α,25-dihydroxy-16-ene-D₃ [1,25(OH)₁₋₁₆₋₁₉⁻νor-D₃] and 1α,25-dihydroxy-16-ene-19-nor-D₃ [1,25(OH)₁₋₁₆₋₁₉⁻νor-D₃] and their 24-oxo metabolites showed greater potency than 1,25(OH)₂D₃ in their abilities to inhibit clonal proliferation of HL-60, NB4, and U937 leukemic cell lines as measured by methylcellulose soft-gel assay. Their inhibition of clonal growth was irreversible as analyzed by pulse exposure studies. The synthetic analogues also had greater potency than 1,25(OH)₂D₃ to induce differentiation of HL-60 and NB4 cells as measured by generation of superoxide, nonspecific esterase production, and induction of CD11b and CD14 cell surface antigens and to increase the proportion of these cells in the G₀-G₁ phase of the cell cycle. For most assays, the 24-oxo metabolite was slightly more potent than the unmodified analogue, and 50% activity was usually found in the nanomolar range. These analogues and their 24-oxo metabolites also inhibited fresh leukemic cell clonal proliferation. Expression of p27KIP₁, a cyclin-dependent kinase inhibitor that plays an important role in blocking the cell cycle, was found by Western blot analysis to be induced by the analogues and their 24-oxo metabolites in both HL-60 and U937 cells, suggesting a possible mechanism by which these analogues inhibit leukemic growth. Notably, the calcemic activity tested by injections of 1α,25-dihydroxy-16-ene-24-oxo-19-nor-D₃ in mice was at least 12-fold less than 1α,25(OH)₁₋₁₆₋₁₉⁻νor-D₃. Taken together, chemically synthesized 24-oxo metabolites of 1α,25(OH)₁₋₁₆₋νor-D₃ and 1α,25(OH)₁₋₁₆₋₁₉⁻νor-D₃ irreversibly inhibited proliferation and induced differentiation of acute myeloid leukemia cells with minimal toxicity; these compounds may have a role in the maintenance phase of therapy for acute myeloid leukemia.

ABSTRACT

The seco-steroid hormone, 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] inhibits proliferation and induces differentiation of malignant cells including those of the hematopoietic system. The 24-oxo metabolite of 1,25(OH)₂D₃ also has prominent antiproliferative activities against various cancer cells. We chemically synthesized five novel 24-oxo vitamin D₃ analogues and evaluated their abilities both to inhibit clonal growth and induce differentiation of myeloid leukemia cells and to cause hypercalcemia. The 1α,25-dihydroxy-16-ene-D₃ [1,25(OH)₁₋₁₆₋₁₉⁻νor-D₃] and 1α,25-dihydroxy-16-ene-19-nor-D₃ [1,25(OH)₁₋₁₆₋₁₉⁻νor-D₃] and their 24-oxo metabolites showed greater potency than 1,25(OH)₂D₃ in their abilities to inhibit clonal proliferation of HL-60, NB4, and U937 leukemic cell lines as measured by methylcellulose soft-gel assay. Their inhibition of clonal growth was irreversible as analyzed by pulse exposure studies. The synthetic analogues also had greater potency than 1,25(OH)₂D₃ to induce differentiation of HL-60 and NB4 cells as measured by generation of superoxide, nonspecific esterase production, and induction of CD11b and CD14 cell surface antigens and to increase the proportion of these cells in the G₀-G₁ phase of the cell cycle. For most assays, the 24-oxo metabolite was slightly more potent than the unmodified analogue, and 50% activity was usually found in the nanomolar range. These analogues and their 24-oxo metabolites also inhibited fresh leukemic cell clonal proliferation. Expression of p27KIP₁, a cyclin-dependent kinase inhibitor that plays an important role in blocking the cell cycle, was found by Western blot analysis to be induced by the analogues and their 24-oxo metabolites in both HL-60 and U937 cells, suggesting a possible mechanism by which these analogues inhibit leukemic growth. Notably, the calcemic activity tested by injections of 1α,25-dihydroxy-16-ene-24-oxo-19-nor-D₃ in mice was at least 12-fold less than 1α,25(OH)₁₋₁₆₋₁₉⁻νor-D₃. Taken together, chemically synthesized 24-oxo metabolites of 1α,25(OH)₁₋₁₆₋νor-D₃ and 1α,25(OH)₁₋₁₆₋₁₉⁻νor-D₃ irreversibly inhibited proliferation and induced differentiation of acute myeloid leukemia cells with minimal toxicity; these compounds may have a role in the maintenance phase of therapy for acute myeloid leukemia.

MATERIALS AND METHODS

Cells. The human myeloid leukemia cell lines (HL-60, U937, and KG-1) were obtained from either the American Type Culture Collection (Rockville, MD) or our laboratory (18). The NB4 cells (19) were generously provided by Dr. M. Lanotte (Institut National de la Sante et de la Recherche Medicale, Paris, France). The cells were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% heat-inactivated FCS (HyClone Laboratories, Inc., Logan, UT). Fresh AML cells from either bone marrow or blood intracellular receptors, which are nuclear transcriptional factors of the steroid-thyroid hormone receptor gene superfamily (2). A number of tissues including bone, kidney, and intestine as well as hematopoietic cells contain VDRs (3). The VDR heterodimerizes with RXR (4); and in the presence of 1,25(OH)₂D₃, the complex binds to VDREs contained within the promoter/enhancer region of target genes and thereby regulates specific gene transcription. For example, 1,25(OH)₂D₃ can cause cell cycle arrest at the G₀-G₁ phase in many types of cells. The prominent expression of the CDKIs known as p21WAF₁ and p27KIP₁ can induce G₀-G₁ arrest of the cell cycle. The p21WAF₁ gene contains a VDRE within its promoter region (5). 1,25(OH)₂D₃ can increase the levels of p21WAF₁ and p27KIP₁ in HL-60 and U937 myeloid leukemia cells and in LNCaP prostate cancer cells (6–10). Recently, nongenomic effects of 1,25(OH)₂D₃ have been detected that may be mediated through G proteins, a Ca²⁺ gradient, and/or protein kinase C (10, 11), but these nongenomic activities probably do not affect the cell cycle.

Our previous studies revealed that 1,25(OH)₂D₃ stimulates proliferation of normal myeloid stem cells and induces their differentiation to monocytes/macrophages (12). Furthermore, we and others have demonstrated that 1,25(OH)₂D₃ can inhibit the proliferation of breast, prostate, colon, and hematopoietic cancer cells and enhance the differentiation of malignant hematopoietic cells (12–15). 1,25(OH)₂D₃ also prolongs the survival time of leukemic animals (16). A clinical trial of 1,25(OH)₂D₃ in preleukemia was associated with hypercalcemia at serum concentrations of this seco-steroid that was lower than the optimal antileukemic concentration determined in experiments performed in vitro (17). Thus, we attempted to identify analogues of 1,25(OH)₂D₃ that have strong antiproliferative effects against cancer cells and yet possess diminished capacity to cause hypercalcemia. Recently, we isolated 24-oxo metabolites of potent vitamin D₃ analogues that were formed by a rat kidney perfusion biotransformation system (14). We found that these 24-oxo metabolites, as compared with the parental analogues, had reduced calcemic activity but equally inhibited clonal proliferation of breast and prostate cancer cells and myeloid leukemia cells (14). These findings, although exciting, relied on a laborious method of isolation of these 24-oxo compounds. Furthermore, the kidney biotransformation technique could result in additional modifications of the 24-oxo compounds that were not apparent. In this study, we chemically synthesized a variety of 24-oxo vitamin D₃ analogues and tested their abilities to inhibit the clonal growth and induce the differentiation of the leukemic cell lines HL-60, NB4, U937, and KG-1. Furthermore, the calcemic activity of these 24-oxo analogues was examined.
were obtained at diagnosis after informed consent, and mononuclear cells were separated on Ficoll-Paque (Pharmacia, Piscataway, NJ) gradients at a density of 1.077. The percentage of bone marrow or circulating leukemic blast cells was greater than 95%. The diagnosis and type of leukemia were based on the French-American-British classification of leukemia (20).

**Vitamin D₃ Compounds.** The parental compound [1α,25(OH)₂D₃, vitamin D₃] and the analogues were dissolved in absolute ethanol at 10⁻³ m as a stock solution, which was stored at −20°C and protected from light. The concentration of the analogues was determined via UV absorbance measurements using their molar extinction coefficient at 264 nM. Dilutions were made in the same tissue culture medium used for the growing of cells. The analogues were synthesized by Hoffmann LaRoche Inc. (Nutley, NJ), and the simplified code names and structures of the vitamin D₃ analogues are shown in Fig. 1. The maximal concentration of ethanol used in this study had no influence on cell growth.

**Clonogenic Analysis.** Myeloid leukemia cell lines were cultured at 1 × 10⁵ cells/well in 24-well plates in methycellulose according to previously described methods (21). For analysis of fresh myeloid leukemia cell clonal growth, 2 × 10⁶ blast cells were plated, and vitamin D₃ analogues were added as indicated. After incubation for 10 days, colonies (>40 cells) were counted using an inverted microscope. All experiments were performed using triplicate plates per experimental point; each experiment was performed at least three times. The results were expressed as the percentage of clonal growth in plates containing vitamin D₃ analogues as compared with the number of colonies in control dishes without vitamin D₃ analogues.

**Pulse Exposure Experiments.** The HL-60 and NB4 cells were incubated in liquid culture with 10⁻⁷ m concentrations of either 1,25(OH)₂D₃ or its analogues for various durations. After incubation, these cells were carefully washed twice with PBS, and viable cells were counted and plated at 1 × 10⁴ cells/well in 24-well plates for colony assay.

**Studies of Induction of Differentiation.** NBT reduction was assayed as described previously (21). NSE activity was examined on cyt centrifuge preparations using a kit (Esterase Stain Kit; Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The cells were grown in liquid culture with RPMI 1640 with 10% FCS for 5 days in a humidified atmosphere, 5% CO₂ at 37°C either with or without vitamin D₃ analogues. For analysis of cell surface antigens, the two-color direct immunofluorescence staining technique was used (21). Cells were stained by using phycoerythrin-conjugated murine antihuman CD11b and FITC-conjugated murine antihuman CD14 (both from Dako Corp., Carpinteria, CA). Control studies were performed with nonbinding control murine IgG1 and IgG2a isotype antibodies (Dako). Analysis of fluorescence was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

**Cell Cycle Analysis.** DNA was stained with propidium iodide after a total of 5 × 10⁶ exponentially proliferating cells were either cultured with the vitamin D₃ analogues (10⁻⁷ m for 5 days) or left untreated (control). Total cells were harvested, washed, resuspended in PBS, and fixed in a 2:1 (v/v) ratio in chilled methanol for 1 h before staining with propidium iodide in the presence of RNase One (Promega, Madison, WI). Cell cycle distribution was determined on a Becton Dickinson FACScan Flow Cytometer and CellFIT Cell-Cycle Analysis software.

**Detection of p21WAF1 and p27KIP1 Cellular Content by Immunoblotting.** Western blot analysis was performed as described previously (22). Briefly, an equal number of cells (10⁸) were lysed in radioimmunoprecipitation assay buffer (1% NP-40–0.5% sodium deoxycholate-0.1% SDS) containing the freshly added protease inhibitors apro tin (30 µg/ml), phenylmethylsulfonyl fluoride (100 µg/ml), and sodium o- vanadate (100 µM). After the amount of protein was equalized by a fluorescence protein assay (Bio-Rad Laboratories, Hercules, CA), cell lysates (30 µg/lane) were mixed with an equal amount of sample buffer (125 mM Tris-HCl, pH 6.8–4.0% SDS-20% glycerol- 10% β- mercaptoethanol-0.02% bromphenol blue), denatured for 90 s at 100°C, and electrophoresed on a 12% SDS-polyacylamide gel. Proteins were electrophoretically transferred onto Immobilon membranes (Millipore Corp., Bedford, MA). Blocking was performed for 1 h with Tris-buffered saline including 0.05% Tween 20 and 2% nonfat dry milk. The membranes were incubated for 1 h with primary monoclonal murine antihuman p21WAF1 antibody (Calbiochem, Cambridge, MA), murine antihuman p27KIP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and murine antihuman β-tubulin antibody (Boehringer Mannheim Biochemica, Mannheim, Germany; 1:100). After two washes with Tris-buffered saline-0.05% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated sheep antip murine IgG (Amersham Corp., Arlington Heights, IL). After four subsequent washes, the bound antibody was visualized with enhanced chemiluminescence reagents (Amersham Corp.), and the membranes were immediately exposed to X-ray film. Densitometry was performed to quantify changes in detected protein.

**Serum Calcium Levels in Vivo.** The major side effect of vitamin D₃ analogues is hypercalcemia. Analysis of the calcemic potency is very relevant when choosing a suitable vitamin D₃ analogue for clinical trials. We compared the hypercalcemic effects of compounds 3 and 3m (Fig. 1). Ninety-six male BALB/c mice at 8–9 weeks of age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), maintained in pathogen-free conditions, and fed a standard laboratory diet. Four mice per group were injected i.p. every other day (except Saturday and Sunday) with either vitamin D₃ analogues or diluent (100 µl/mouse) for 3 weeks. Doses of compounds 3 and 3m were 0.5, 1.0, 3.0, and 6.0 µg/mouse. Moreover, we injected compound 3 (6.0 µg/mouse) i.p. for 3 weeks to wild-type mice having an intact vitamin D₃ receptor (VDR⁺/ᵐ) and to mice with homozygous germline deletion of VDR (VDR⁻/⁻), respectively, and compared serum calcium values, which were measured every week by the quantitative, colorimetric detection assay using the Sigma 587 kit (Sigma, St. Louis, MO).

**RESULTS**

**Effects of 24-Oxo-substituted Vitamin D₃ Analogues: Inhibition of Clonal Growth of Myeloid Leukemia Cells.** The 1,25(OH)₂D₃ (brief name, compound C) inhibited clonal growth of three of the acute myeloid leukemia cell lines in a dose-response manner (Fig. 2). The effective dose that inhibited 50% colony formation (ED₅₀) of HL-60, NB4, and U937 was ~10, 8, and 12 × 10⁻⁹ M, respectively (Table 1). The clonal growth of KG-1 leukemia cells was not inhibited...
in the presence of compound C. Previous studies by ourselves have shown that 1,25(OH)₂-D₃ (compound 2) was a potent inhibitor of clonal growth of myeloid leukemia cells (14). We compared its inhibitory activity to a probable intracellular metabolite of this agent, 1,25(OH)₂-16-ene-24-oxo-D₃ (compound 2m). The 1,25(OH)₂-16-ene-24-oxo-D₃ was as potent as or more potent than the 1,25(OH)₂-16-ene-D₃ analogue (Fig. 2; Table 2) with the former having an ED₅₀ of 0.2, 0.2, and 0.07 × 10⁻⁹ M for HL-60, NB4, and U937, respectively. Thus, the 24-oxo compound was 40–170-fold more potent than the 1,25(OH)₂-D₃ depending on which leukemia cell line was examined. Furthermore, another 24-oxo metabolite [1,25(OH)₂-16-ene-24-oxo-19-nor-D₃, compound 3m] was slightly more potent than the same analogue without the 24-oxo motif (compound 3). Both had a range of potencies similar to those of compound 2 and 2m (Fig.

![Fig. 2. Dose-response effects of vitamin D₃ (VD) compounds on clonal proliferation of HL-60, NB4, U937, and KG-1 myeloid leukemia cells. Results are expressed as a mean percentage of control plates containing no vitamin D₃ compounds. Each data point represents a mean of at least three independent experiments with triplicate dishes. Bars, SD.](image)

Table 1  **Effect of vitamin D₃ analogues on cellular proliferation of myeloid leukemia cells**

<table>
<thead>
<tr>
<th>Chemical name of analogues</th>
<th>Code</th>
<th>HL-60 ED₅₀ (×10⁻⁹ M)</th>
<th>NB4</th>
<th>U937</th>
<th>KG-1</th>
</tr>
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<tbody>
<tr>
<td>1α,25-(OH)₂-D₃</td>
<td>C</td>
<td>10</td>
<td>8</td>
<td>12</td>
<td>NR</td>
</tr>
<tr>
<td>1α,25-(OH)₂-16-ene-D₃</td>
<td>2</td>
<td>0.8</td>
<td>0.7</td>
<td>0.07</td>
<td>NR</td>
</tr>
<tr>
<td>1α,25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>2m</td>
<td>0.2</td>
<td>0.2</td>
<td>0.07</td>
<td>NR</td>
</tr>
<tr>
<td>1α,25-(OH)₂-16-ene-19-nor-D₃</td>
<td>3</td>
<td>0.8</td>
<td>0.8</td>
<td>0.08</td>
<td>NR</td>
</tr>
<tr>
<td>1α,25-(OH)₂-16-ene-24-oxo-19-nor-D₃</td>
<td>3m</td>
<td>0.1</td>
<td>0.1</td>
<td>0.06</td>
<td>NR</td>
</tr>
<tr>
<td>1-Fluoro-25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>4</td>
<td>10</td>
<td>80</td>
<td>7</td>
<td>NR</td>
</tr>
<tr>
<td>3-Deoxy-1α,25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>5</td>
<td>400</td>
<td>100</td>
<td>80</td>
<td>NR</td>
</tr>
<tr>
<td>25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>6</td>
<td>NR</td>
<td>37</td>
<td>800</td>
<td>NR</td>
</tr>
</tbody>
</table>

* (OH), hydroxy; (OH)₂, dihydroxy; D₃, vitamin D₃; NR, not reactive (ED₅₀ was not reached even at 10⁻⁶ M concentrations of the vitamin D₃ compound); ND, not determined.

Table 2  **Cell surface markers by HL-60, NB4, and U937 cells cultured with vitamin D₃ analogues**

HL-60, NB4, and U937 cells were cultured in liquid medium with vitamin D₃ analogues (10⁻⁸ M, 96 h). Results given as percentage of positive cells and FI in arbitrary units.

<table>
<thead>
<tr>
<th>Code</th>
<th>Vitamin D₃ analogues</th>
<th>HL-60</th>
<th>NB4</th>
<th>U937</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD11b</td>
<td>CD14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td>FI</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>3</td>
<td>33.0</td>
<td>2</td>
</tr>
<tr>
<td>1α,25-(OH)₂-D₃</td>
<td>1</td>
<td>55</td>
<td>91.1</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>1α,25-(OH)₂-16-ene-D₃</td>
<td>76</td>
<td>126.3</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>1α,25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>84</td>
<td>157.1</td>
<td>65</td>
</tr>
<tr>
<td>3m</td>
<td>1α,25-(OH)₂-16-ene-19-nor-D₃</td>
<td>86</td>
<td>161.7</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>1-Fluoro-25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>91</td>
<td>180.0</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>3-Deoxy-1α,25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>48</td>
<td>112.4</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>25-(OH)₂-16-ene-24-oxo-D₃</td>
<td>31</td>
<td>66.7</td>
<td>25</td>
</tr>
</tbody>
</table>

* (OH)₂, dehydroxy; (OH), hydroxy; D₃, vitamin D₃.
Table 2). The clonal growth of KG-1 was not inhibited by any of these compounds.

Three additional 24-oxo compounds were examined. One had the 1α-hydroxyl substituted with a 1-fluoro motif [1-fluoro-25-dihydroxy-16-ene-24-oxo-vitamin D₃, compound 4]. The second had a 3-deoxy moiety added to 1,25(OH)₂-16-ene-24-oxo-D₃ (compound 5). The third analogue had the 1α-hydroxyl removed from the 1,25(OH)₂-16-ene-19-nor-D₃ (compound 6). Each of these substitutions decreased the activity of the parental compound (Table 1).

Additional experiments focused on the most active vitamin D₃ analogues and their 24-oxo metabolites: compounds 2, 2m, 3, and 3m as well as 1,25(OH)₂D₃ (compound C). Fresh AML cells were enriched from individuals with newly diagnosed leukemia, and the ability of the vitamin D₃ analogues to inhibit their clonal growth was investigated (Fig. 3). All four analogues at each concentration were more potent than 1,25(OH)₂D₃. For samples 1 (French-American-British classification M2), 2 (M2), and 4 (M4), the potency of each of the analogues was very similar, with ED₅₀ so, 10⁻²⁸–3, 10⁻²³ M; 1,25(OH)₂D₃ was 10-fold less potent. For the AML sample from individual 3 (M2), 1,25(OH)₂D₃ was as potent as the vitamin D₃ analogues (ED₅₀ 5, 3, 10⁻²⁸ M, Fig. 3).

### Effects of Vitamin D₃ Analogues on Differentiation of Myeloid Leukemia Cells

Differentiation was examined using several well-recognized parameters of myeloid maturation. The NBT assay measures superoxide production and becomes positive as a cell differentiates along either the monocytic or granulocytic pathway (21). The 1,25(OH)₂D₃ as well as each of the analogues and their 24-oxo derivatives markedly enhanced NBT reduction by HL-60 cells (Fig. 4). For example, exposure to these compounds (10⁻²⁻⁷ m, 5 days) produced about a 4-fold increase in NBT activity. The NB4 cells had a similar response, albeit these cells produced greater positivity at a lower concentration of the vitamin D₃ analogues.

Another parameter of monocytic differentiation is the development of nonspecific acid esterase activity (NSE) which can be identified by staining for this enzyme. Each of the analogues had nearly the same activity as 1,25(OH)₂D₃, and ~50% of HL-60 cells were positive at 10⁻⁻⁷ m concentrations of each of the compounds. Two additional markers of myeloid differentiation are CD11b (β integrin subunit, expressed by both granulocytes and monocytes) and CD14 (late monocytic cell surface marker). About 3% (FI 33.0) and 2% (FI 9.9) of untreated HL-60 cells expressed CD11b and CD14 cell surface antigens, respectively (Table 2). Expression of these cell surface antigens increased with exposure to 1,25(OH)₂D₃ (10⁻⁻⁸ m, 96 h), with 55% (FI 91.1) and 35% (FI 44.4) of the HL-60 cells induced to express CD11b and CD14, respectively. Compounds 2, 2m, 3, and 3m induced expression of CD11b in HL-60, NB4, U937, and CD14 in HL-60 and U937 cells as reflected by increase of FI per cell as compared with cells cultured with 1,25(OH)₂D₃. The potency of the 24-oxo metabolites was slightly greater than that of their parental compounds for both measurements of differentiation for both cell lines. In contrast, compounds 4, 5, and 6 had the same or less potency compared with 1,25(OH)₂D₃ to induce CD11b and CD14 expression in these cells.

Fig. 3. Effect of 1,25(OH)₂D₃ and its analogues on clonal proliferation of leukemic cells from four patients with acute myeloid leukemia. Results are expressed as a percentage of control plates containing no vitamin D₃ (VD) compounds. Bars, SD.

Fig. 4. Induction of differentiation of HL-60 and NB4 cells, as measured by their abilities to reduce NBT and stain with NSE. HL-60 and NB4 cells were treated with 1,25(OH)₂D₃ and its analogues (10⁻⁻¹⁰⁻⁻¹⁰⁻⁸ m, 5 days), and differentiation was determined by NBT reduction and NSE staining. Results represent mean ± SD of three independent experiments with triplicate dishes. VD, vitamin D₃.
Effects of Pulse Exposure to Vitamin D₃ Analogues. To examine the effects of pulse exposure of vitamin D₃ analogues on clonogenic growth of HL-60 and NB4, these cells were cultured in liquid for 2–5 days with 10⁻⁷ m vitamin D₃ and its analogues. The cells were extensively washed to remove the vitamin D₃ compounds, and the viable cells were counted (>95% viable) and plated in methylcellulose culture; colonies were enumerated after 10 days of culture. Results are expressed as a mean percentage of colonies in control plates containing no compounds. Each point represents a mean ± SD of at least three experiments with each experimental point having triplicate dishes.

Effects of Vitamin D₃ Analogues on Cell Cycle Distribution of HL-60 and NB4 Cells. Effects of vitamin D₃ analogues and their 24-oxo-containing compounds (10⁻⁷ m, 5 days) on the cell cycle distribution of HL-60 and NB4 cells were studied (Table 3). In each case, all of the vitamin D₃ compounds markedly and significantly increased the percentage of cells in the G₀-G₁ phase. For example, 48 and 57% of wild-type HL-60 and NB4, respectively, were in G₀-G₁; after exposure to 1,25(OH)₂D₃, the percentage of cells in G₀-G₁ increased to 76 and 79%, respectively. The analogues and their 24-oxo metabolites gave similar or slightly higher numbers. P values for the analogues and their 24-oxo metabolites were very similar (Table 3). Concomitant with these changes, the percentage of cells in S phase decreased (Table 3). The number of wild-type HL-60 and NB4 cells in S phase was 33 and 26%, respectively, which decreased to 12% in those that received 1,25(OH)₂D₃. The analogues and their 24-oxo metabolites resulted in similar or lower percentage of HL-60 and NB4 cells in S phase.

Induction of Expression of p21(WAF1) and p27(KIP1) by Vitamin D₃ Analogues. The p21(WAF1) and p27(KIP1) are CDKIs that bind to complexes of cyclin and cyclin-dependent kinase and decrease the kinase activity usually associated with an increased percentage of cells in G₀-G₁ and/or G₂ (23–25). Prior experiments have shown that maximal expression of p27(KIP1) in HL-60 cells exposed to 1,25(OH)₂D₃ occurs at ~96 h (26). Therefore, HL-60 and U937 cells were cultured with various vitamin D₃ analogues (10⁻⁷ m) for 96 h and analyzed for changes in protein expression of p21(WAF1) and p27(KIP1) using Western blot (Fig. 6). HL-60 cells constitutively had a very low level of p21(WAF1); and none of the analogues induced the expression of p27(KIP1). On the other hand, U937 cells expressed negligible level of p21(WAF1); and the vitamin D₃ analogues slightly induced p21(WAF1) expression. The untreated U937 cells expressed low level of p27(KIP1), and the expression increased by 3.3- to 4.3-fold after exposure to these analogues.

Effects of Vitamin D₃ Analogues on Serum Calcium Levels. Because hypercalcemia is a major toxicity of vitamin D₃ compounds, the calcemic effects of compounds 3 and 3m were compared (Fig. 7). The mice that received 1.0 μg of compound 3 (three times per week) I.P. were hypercalcemic, with mean serum calcium levels of ~12.1 ± 0.7 mg/dl (normal, 8.5–10.5 mg/dl) at week 3. When 3.0 and 6.0 μg/mouse compound 3 were administered, serum calcium levels increased to means of 14.0 ± 0.5 and 18.5 ± 1.1 mg/dl at week 3, respectively. In contrast, mice that received compound 3m (0.5–6.0 μg/mouse) did not show hypercalcemia (9.5–10.4 mg/dl at week 3). We wanted to determine whether the vitamin D₃ analogues were mediating their activity through the classic VDR genomic pathway. Therefore, we treated wild-type (VDR⁻/⁻) and VDR knockout (VDR⁻/-) mice with 6.0 μg of compound 3 for 3 weeks (Fig. 7). When VDR⁻/⁻ mice received 6.0 μg three times per week of compound 3, the serum calcium levels increased from 8.9 to 15.0–15.5

Table 3 Cell cycle changes mediated by vitamin D₃ analogues and their 24-oxo metabolites

<table>
<thead>
<tr>
<th>Vitamin D₃ compounds</th>
<th>Control</th>
<th>1α,25(OH)₂D₃</th>
<th>16-ene</th>
<th>16-ene-24-oxo</th>
<th>16-ene-19-nor</th>
<th>16-ene-24-oxo-19-nor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>G₀-G₁</td>
<td>48.4 ± 1.7</td>
<td>76.6 ± 0.9⁷</td>
<td>82.3 ± 1.0⁷</td>
<td>83.6 ± 1.5⁷</td>
<td>83.9 ± 1.0⁷</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>33.1 ± 1.6</td>
<td>12.5 ± 1.3⁷</td>
<td>9.9 ± 0.7⁷</td>
<td>9.0 ± 0.9⁷</td>
<td>9.0 ± 0.8⁷</td>
</tr>
<tr>
<td></td>
<td>G₀-M</td>
<td>18.5 ± 0.7</td>
<td>10.8 ± 1.3⁷</td>
<td>7.8 ± 0.5⁷</td>
<td>7.4 ± 0.6⁷</td>
<td>7.0 ± 0.4⁷</td>
</tr>
<tr>
<td>NB4</td>
<td>G₀-G₁</td>
<td>57.6 ± 1.5</td>
<td>79.8 ± 1.5⁷</td>
<td>82.5 ± 1.3⁷</td>
<td>83.3 ± 1.3⁷</td>
<td>82.2 ± 1.2⁷</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>26.1 ± 1.1</td>
<td>12.5 ± 1.4⁷</td>
<td>9.9 ± 1.0⁷</td>
<td>10.4 ± 1.3⁷</td>
<td>10.3 ± 0.7⁷</td>
</tr>
<tr>
<td></td>
<td>G₀-M</td>
<td>16.3 ± 0.4</td>
<td>7.7 ± 0.3⁷</td>
<td>7.6 ± 0.4⁷</td>
<td>6.3 ± 0.3⁷</td>
<td>7.5 ± 0.6⁷</td>
</tr>
</tbody>
</table>

* P < 0.0001.
* P < 0.001.
epi-vitamin D₃] inhibited clonal proliferation of prostate and breast cancer cell lines as well as the HL-60 myeloid leukemia cell line, and this activity was equivalent to the parental analogues (14). Furthermore, the natural 24-oxo metabolites appeared to cause less calcemic effects than the parental compounds. The biological synthesis and purification of "natural" 24-oxo metabolites are cumbersome, are inefficient, and could result in unexpected modifications.

To overcome these difficulties, we chemically synthesized the 24-oxo metabolite of several of our lead vitamin D₃ analogues: 1.25(OH)₂-16-ene-D₃ and 1.25(OH)₂-16-ene-19-nor-D₃. Both of the analogues and their 24-oxo metabolites had a remarkable ability to inhibit clonal growth of HL-60, NB4, and U937 cells as well as fresh myeloid leukemia cells. These analogues irreversibly inhibited the growth of HL-60 and NB4 cells as shown by pulse exposure studies. Moreover, these compounds efficiently induced differentiation of HL-60, NB4, and U937 cells as measured by NBT reduction, NSE activity, and CD11b and CD14 cell surface antigen expression. Cell cycle analysis of HL-60 and NB4 cells exposed to these vitamin D₃ analogues and their 24-oxo metabolites possessed similar potencies to induce a G₁ arrest of HL-60 and NB4 cells. The analogues and their 24-oxo metabolites were more potent than 1,25(OH)₂D₃ for most of the studies. Prior studies have shown that the clonogenic assay was more sensitive than the differentiation assays (28). The same was found in this study. The clonogenic assay requires the cells to proliferate at least five times to form a colony large enough to be scored. The vitamin D₃ compounds could inhibit growth by several processes including apoptosis or inducing the cells to terminally differentiate. The measurement of NBT positivity is not a very sensitive measurement and becomes positive only with differentiation of the cells.

We have previously found that 1,25(OH)₂D₃ did not inhibit clonal growth of KG-1 cells (29). Congruent with this finding, we have found that none of the vitamin D₃ analogues inhibited growth of these cells. The mechanism by which these cells are resistant is unclear. They do not have a detectable mutation of the VDR (30). These cells do not have a detectable mutation of the VDR (30). These cells are very immature and clearly do not differentiate with a variety of vitamin D₃ compounds or other differentiative compounds. This is in contrast to the other lines used in this study, HL-60, U937, and NB4.

Previously, we showed that mice can receive no more than about 0.1–0.2 μg of 1,25(OH)₂D₃ (three times per week) without developing fatal hypercalcemia (27). A remarkable finding of this study is that 1,25(OH)₂-16-ene-24-oxo-19-nor-D₃ (compound 3m) did not induce hypercalcemia, even at the extremely high dose of 6 μg, three times per week. The same high dose of 1,25(OH)₂-16-ene-19-nor-D₃ (compound 3) without the 24-oxo motif produced life-threatening hypercalcemia. Thus, the addition of the 24-oxo motif produced life-threatening hypercalcemia. The biological synthesis and purification of these analogues plays an important role in reducing the calcemic activity of the vitamin D₃ analogues.

**DISCUSSION**

We previously showed that vitamin D₃ analogues that have desaturation at C-16 have prominent antiproliferative activity against a variety of cancer cells (27). Furthermore, removal of the C-19 in conjunction with desaturation of C-16 resulted in an active analogue that inhibited the growth of prostate and breast cancer cells as well as leukemia cells (26). Using a kidney perfusion model, we found that enzymatically produced, natural 24-oxo-vitamin D₃ metabolites of the vitamin D₃ analogues [1,25(OH)₂-16-ene-D₃ and 1,25-dihydroxy-20-
Why does the 24-oxo metabolites have the same antiproliferative and prodifferentiative effects as other vitamin D analogues without causing hypercalcemia? This query requires additional studies. Perhaps the 24-oxo analogue interacts differently with the VDR/RXR complex in transformed hematopoietic cells than it does with the intestinal cells. Precedence for this phenomenon occurs with the selective estrogen receptor modifiers. For example, tamoxifen is an antagonist of the estrogen receptor for breast, vagina, and central nervous system tissue, but it acts as an agonist for bone, endometrium, and lipid metabolism (31). Furthermore, raloxifene is another selective estrogen receptor modifier but acts as an estrogen receptor antagonist rather than an agonist in the endometrium (31). Possibility, these selected ligands can alter their cognate nuclear hormone receptors so that their interaction with coactivators and corepressors is modified (31–33). Thus, the VDR complex bound to the 24-oxo vitamin D3 ligands may be inducing coactivators to mediate transactivation of target gene in hematopoietic cells, and these same complexes in the intestinal cells may bind corepressors to block the transcriptional activity of the receptor. 

Experiments in vitro suggest that vitamin D3 analogues might mediate some of their activity through a nongenomic pathway. For example, these compounds might bind to a cell surface receptor resulting in a cascade of secondary signals producing a biological response such as calcium influx. However, at least for calcium homeostasis, this probably is unlikely. When we administered 1,25(OH)2-16-ene-19-nor-D3 to wild-type mice, they developed hypercalcemia. However, when the same dose was given to mice that had homozygous germline deletion (knockout) of VDR, they did not develop hypercalcemia. This strongly suggests that the vitamin D3 compounds control calcium absorption from the intestine and reabsorption from the bone by the classic ligand/VDR/VDRE pathway.

The mechanism by which the vitamin D3 analogues inhibit the proliferation of the myeloid leukemia cells is also unknown. The vitamin D3 compounds are capable of arresting cells in G1. The CDKIs are key regulators of the cell cycle. Expression of the p27kip1 CDKI was up-regulated 3.0–3.2-fold by 1,25(OH)2D3, 1α,25(OH)2-16-ene-D3, 1α,25-(OH)2-16-ene-19-nor-D3, and their 24-oxo metabolites in HL-60 cells. This CDKI was also up-regulated by these compounds in U937 cells; thus, up-regulation of p27kip1 may be one of the mechanisms that mediate the cell cycle arrest. Expression of another CDKI, p21WAF1, was up-regulated by these vitamin D3 compounds in U937 cells but not in HL-60 cells, suggesting that the contribution of p21WAF1 to the cell cycle arrest mediated by vitamin D3 compounds varies depending on the myeloid cells. The mechanism for this divergence is unclear. The upstream region of p21WAF1 has a VDRE, but this clearly is not enough to assure a response to the vitamin D3 compounds. Paradigmatically, the upstream region of p27kip1 does not have an obvious VDRE. Taken together, the data suggest the need for further studies.

The goal of this research is to develop a vitamin D3 analogue that has anticancer activity with little capacity to cause side effects such as hypercalcemia (34). The 1,25(OH)2-16-ene-24-oxo-19-nor-D3 may come closest to this goal. The analogue is at least 12-fold less able to induce hypercalcemia than is 1,25(OH)2D3 (35), but it has ~100-fold increased capacity to inhibit the clonal proliferation of leukemia cell lines. Clearly, these observations must be extended to in vivo cancer models as well as to other cancer types. Realistically, vitamin D3 analogues may have a role in the adjuvant or maintenance setting or in combination with chemotherapeutic agents, because of their low, non-cross-reactive toxicity. They are unlikely to be used as a sole therapeutic modality. On a molar basis, the vitamin D3 analogues are more potent than chemotherapeutic drugs such as daunorubicin if their antiproliferative ED50 activities are compared (36, 37). However, the vitamin D3 analogues appear often to have difficulty inhibiting the last few percentages of the malignant population of cells. Furthermore, some transformed cells are very resistant to the vitamin D3 analogues, as shown by the refractory KG-1 cells.


24-Oxo Metabolites of Vitamin D₃ Analogue: Disassociation of Their Prominent Antileukemic Effects from Their Lack of Calcium Modulation

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