Novel vitamin D₃ analog, 21-(3-methyl-3-hydroxy-butyl)-19-nor D₃, that modulates cell growth, differentiation, apoptosis, cell cycle, and induction of PTEN in leukemic cells

Jun-ichi Hisatake, James O’Kelly, Milan R. Uskokovic, Shigeru Tomoyasu, and H. Phillip Koeffler

The active form of vitamin D₃, 1,25(OH)₂D₃, inhibits proliferation and induces differentiation of a variety of malignant cells. A new class of vitamin D₃ analogs, having 2 identical side chains attached to carbon-20, was synthesized and the anticancer effects evaluated. Four analogs were evaluated for their ability to inhibit growth of myeloid leukemia (NB4, HL-60), breast (MCF-7), and prostate (LNCaP) cancer cells. All 4 analogs inhibited growth in a dose-dependent manner. Most effective was 21-(3-methyl-3-hydroxy-butyl)-19-nor D₃ (Gemini-19-nor), which has 2 side chains and removal of the C-19. Gemini-19-nor was approximately 20% of cells, whereas 1,25(OH)₂D₃ at the same concentration did not induce apoptosis. Gemini-19-nor increased in HL-60 both the proportion of cells in the G₁/G₀ phase and expression level of p27kip1. Moreover, Gemini-19-nor stimulated expression of the potential tumor suppressor, PTEN.

Introduction

The present chemotherapy of cancer uses agents that are usually toxic to normal cells. On the other hand, induction of cellular differentiation may supplement the use of cytotoxic drugs in several forms of neoplasia, like the successful use of all-trans-retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia. The physiologically active form of vitamin D₃, 1,25(OH)₂D₃, is a member of the secosteroid hormone family, which controls calcium homeostasis and bone metabolism. 1,25(OH)₂D₃ can induce differentiation and inhibit the growth of a number of malignant cell types, including myeloid leukemia, breast, prostate, colon, skin, and brain. Several studies suggested that growth inhibition by 1,25(OH)₂D₃ may be attributed to inhibition of the G₁ to S transition in the cell cycle, which probably is due at least in part to stimulation of expression of the cyclin-dependent kinase inhibitors (CDKIs), p21waf1 and p27kip1 as well as induction of programmed cell death. In a clinical study, oral administration of 1,25(OH)₂D₃ to preleukemic patients was only partially effective: calcemic side effects prevented the administration of the dosage of the compound needed to achieve 50% clonal growth (ED₅₀) of NB4, HL-60, MCF-7, and LNCaP cells, respectively. Gemini-19-nor (10⁻⁸ M) strongly induced expression of CD11b and CD14 on HL-60 cells (90%); in contrast, 1,25(OH)₂D₃ (10⁻⁸ M) stimulated only 50% expression. Annexin V assay showed that Gemini-19-nor and 1,25(OH)₂D₃ induced apoptosis in a dose-dependent fashion. Gemini-19-nor (10⁻⁸ M, 4 days) caused apoptosis in approximately 20% of cells, whereas 1,25(OH)₂D₃ at the same concentration did not induce apoptosis. Gemini-19-nor increased in HL-60 both the proportion of cells in the G₁/G₀ phase and expression level of p27kip1. Therefore, synthesis of vitamin D₃ analogs with potent antiproliferative and differentiation activity against cancer cells with decreased risk of hypercalcemia has received considerable attention.

Recently, PTEN/MMAC1/TEP1, a tyrosine phosphatase, was identified and mapped to chromosome 10q23.3. PTEN gene mutations have been observed in a variety of human cancers including breast, prostate, brain, lymphoma, and leukemia. Germline deletion of PTEN in the mouse resulted in early embryonic lethality, and heterozygous mice developed malignant neoplasms. These findings strongly suggested that PTEN is a candidate tumor suppressor.

In this study, a class of newly synthesized vitamin D₃ analogs having 2 identical side chains attached to carbon-20 was analyzed. We focused particularly on the most active analog, which has a deletion of C-19, 21-(3-methyl-3-hydroxy-butyl)-19-nor D₃ (Gemini-19-nor). This new vitamin D₃ analog was more potent than 1,25(OH)₂D₃ in mediating growth inhibition, differentiation, apoptosis, G₁/G₀ arrest of the cell cycle, and expression of p27kip1. Furthermore, we observed that this compound induced the expression of PTEN in myeloid leukemic cells as the cells underwent differentiation.

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Materials and methods

Cells and compounds

The myeloid leukemia (HL-60), breast cancer (MCF-7), and prostate cancer (LNCaP) cell lines were obtained from American Type Culture Collection (Rockville, MD). The NB4 promyelocytic leukemia cell line was provided by Dr Lanotte (INSERM, Hospital Saint-Louis, Paris, France). HL-60, NB4, and LNCaP were cultured in RPMI 1640 with 10% fetal calf serum (FCS). MCF-7 cells were maintained in Dulbecco modified Eagle media with 10% FCS. All 4 cell lines were maintained in a 37°C incubator containing 5% CO2. After informed consent, mononuclear cells from normal bone marrow were collected by separation on Ficoll-Hypaque gradients at a density of 1.077, and washed in Iscove modified Dulbecco medium (IMDM) containing 10% FCS.

All vitamin D3 analogs were synthesized by Milan R. Uskokovic (Hoffmann-La Roche, Nutley, NJ). The analogs are shown in Figure 1. The vitamin D3 compounds were dissolved in absolute ethanol at 10−3 M as stock solution, which were stored at −20°C and protected from light.

Soft agar colony assay

Cells were cultured in a 2-layer soft agar system for either 7 days (HL-60 and NB4) or 10 days (MCF-7 and LNCaP) as described previously.26 Cells were cultured in a 2-layer soft agar system for either 7 days (HL-60) or 10 days (MCF-7 and LNCaP) as described previously.26 Normal bone marrow cells were cultured for 14 days in methylcellulose medium M3234 (Stem Cell Technology, Vancouver, BC, Canada) containing 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). MCF-7 and LNCaP cells were trypsinized, washed, counted, and plated into 24-well, flat-bottom plates with a total of 1 x 105 cells/well in a volume of 400 µL/well. The feeder layer was prepared with agar that had been equilibrated at 42°C. Prior to this step, vitamin D3 compounds were pipetted into the wells. After incubation, the colonies were counted. All experiments were done at least 3 times using triplicate plates per experimental point.

Analysis of differentiation

Expression of cell surface antigens was determined by flow cytometry. HL-60 cells were cultured with either 1,25(OH)2D3 or Gemini-19-nor (10−8 and 10−7 M) for 4 days. After twice washing with phosphate-buffered saline (PBS), cells were incubated for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated murine antihuman CD11b or antihuman CD14 antibody (DAKO, Carpinteria, CA). Murine IgG1 antibody (DAKO) was used as negative control. Cells were analyzed by a FACSscan (Becton Dickinson, Mountain View, CA). HL-60 cells were assessed for their ability to produce superoxide as measured by reduction of nitroblue tetrazolium (NBT), by morphology as detected on cytospin preparations stained with Diff-Quick Stain Set (Baxter Healthcare, Miami, FL). All experiments were independently done at least 3 times. All data were statistically analyzed by Student t test.

Cell cycle analysis

Cell cycle analysis was performed on HL-60 cells incubated for 4 days with either 1,25(OH)2D3 or Gemini-19-nor at either 10−8 or 10−7 M. The cells were fixed in chilled methanol overnight before staining with 50 µg/mL propidium iodide (PI), 1 mg/mL RNase, and 0.1% NP40. Analysis was performed immediately after staining using a FACSscan (Becton Dickinson) and CELLFit program (Becton Dickinson). All experiments were independently performed at least 3 times. All data were statistically analyzed by Student t test.

Apoptosis analysis

To study induction of apoptosis by vitamin D3 analogs, annexin V assay (Annexin V-FITC Apoptosis Detection Kit; Pharmingen, San Diego, CA) was performed according to the manufacturer’s instructions. Briefly, cells were harvested after exposure with either 1,25(OH)2D3 or Gemini-19-nor (10−8 and 10−7 M), washed twice with PBS, incubated with FITC-conjugated annexin V and PI for 15 minutes, and measured by FACSscan (Becton Dickinson). All experiments were independently done at least 3 times. All data were statistically analyzed by Student t test.

Western blot analysis

Cells were washed twice in PBS, suspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% NP40, 100 µg/mL phenylmethylsulfonyl fluoride, 2 µg/mL aprotinin, 1 µg/mL pepstatin, and 10 µg/mL leupeptin), and placed on ice for 30 minutes. After centrifugation at 15 000 g for 20 minutes at 4°C, the supernatant was collected. Protein concentrations were quantitated using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Whole lysates (40 µg) were resolved by 4% to 15% SDS-polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights, IL) and probed with anti-p27kip1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PTEN antibody (Santa Cruz Biotechnology), and anti-GAPDH antibody (Research Diagnostics, Flanders, NJ). The blots were developed using the enhanced chemoluminescence (ECL) kit (Amersham). Band intensity was measured using a densitometer and fold increase in expression as compared to control, untreated cells was calculated.

Results

Effect of vitamin D3 analogs on clonal proliferation

The myeloid leukemia (HL-60 and NB4), breast cancer (MCF-7), and prostate cancer (LNCaP) cells were cloned in soft agar in the
presence of various concentrations of vitamin D$_3$ analogs. All 4 Gemini vitamin D$_3$ analogs and 1,25(OH)$_2$D$_3$ inhibited clonal growth of all 4 cell lines in a dose-dependent manner (Figure 2). The effective dose that inhibited 50% colony formation (ED$_{50}$) was determined (Table 1). Gemini-19-nor was approximately 70-, 40 625-, 23-, and 380-fold more potent than 1,25(OH)$_2$D$_3$ in mediating clonal growth inhibition of HL-60, NB4, MCF-7, and LNCaP cells, respectively. Gemini-1-F-25-OH was 3- to 6-fold stronger than 1,25(OH)$_2$D$_3$ in suppressing clonal growth of the myeloid leukemia cell lines (HL-60 and NB4), but it had the same potency as 1,25(OH)$_2$D$_3$ against MCF-7 and LNCaP cells. Furthermore, the potency of Gemini 5,6-trans and Gemini 3-3pi were nearly equivalent to 1,25(OH)$_2$D$_3$ for all 4 of the cell lines. Because Gemini-19-nor was found to be the most potent compound, all additional experiments focused on this analog.

Clonal growth of normal bone marrow-committed myeloid stem cells (colony-forming units-granulocyte/macrophage [CFU-GM]) were not inhibited by either 1,25(OH)$_2$D$_3$ or Gemini-19-nor over a concentration range of 10$^{-10}$ M to 10$^{-8}$ M (Figure 3). At 10$^{-7}$ M, both compounds inhibited by 20% to 40% the clonal growth of normal bone marrow CFU-GM.

### Table 1. Inhibition of clonal proliferation of cancer cell lines by vitamin D$_3$ analogs

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>1,25(OH)$_2$D$_3$</th>
<th>Gemini-19-nor</th>
<th>Gemini-3-3pi</th>
<th>Gemini-5,6-trans</th>
<th>Gemini-1-F-25-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>1.2 × 10$^{-8}$</td>
<td>1.7 × 10$^{-10}$</td>
<td>2.8 × 10$^{-8}$</td>
<td>1.2 × 10$^{-8}$</td>
<td>4.3 × 10$^{-9}$</td>
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<tr>
<td>NB4</td>
<td>2.6 × 10$^{-8}$</td>
<td>6.4 × 10$^{-13}$</td>
<td>3.9 × 10$^{-8}$</td>
<td>2.1 × 10$^{-8}$</td>
<td>4.0 × 10$^{-9}$</td>
</tr>
<tr>
<td>MCF-7</td>
<td>3.7 × 10$^{-8}$</td>
<td>1.6 × 10$^{-10}$</td>
<td>1.6 × 10$^{-7}$</td>
<td>1.2 × 10$^{-9}$</td>
<td>4.1 × 10$^{-9}$</td>
</tr>
<tr>
<td>LNCaP</td>
<td>× 10$^{-7}$</td>
<td>1.7 × 10$^{-9}$</td>
<td>5.0 × 10$^{-8}$</td>
<td>7.6 × 10$^{-6}$</td>
<td>1.0 × 10$^{-7}$</td>
</tr>
</tbody>
</table>

Results derived from Figure 2: the concentration of vitamin D$_3$ compound that induced 50% clonal inhibition (ED$_{50}$). HL-60, human myeloblastic leukemia cells; NB4, human acute promyelocytic cells; MCF-7, estrogen-receptor positive breast cancer cells; LNCaP, androgen receptor-positive prostate cancer cells.

**Effect of Gemini-19-nor on differentiation of leukemia cell lines**

The induction of expression of the cell surface antigens CD11b and CD14 occurs on HL-60 cells as they undergo differentiation. The ability of Gemini-19-nor and 1,25(OH)$_2$D$_3$ to induce CD11b and CD14 was analyzed using flow cytometry (Figure 4). A 4-day exposure of HL-60 cells to either 1,25(OH)$_2$D$_3$ (10$^{-7}$ M) or Gemini-19-nor (10$^{-7}$ M) resulted in both producing nearly 90% and 80% CD11b$^+$ and CD14$^+$ cells, respectively. At 10$^{-8}$ M, Gemini-19-nor and 1,25(OH)$_2$D$_3$ induced 90% and 50% CD11b$^+$ cells, and 75% and 30% CD14$^+$ cells, respectively.

The capacity of HL-60 cells to produce superoxide as measured by the reduction of NBT was another marker of differentiation that was used. Gemini-19-nor was more potent than 1,25(OH)$_2$D$_3$ with either Gemini-19-nor or 1,25(OH)$_2$D$_3$ (10$^{-8}$ M, 4 days) inducing 70% and 30% NBT$^+$ cells, respectively (Figure 5).

**Effect of Gemini-19-nor on induction of apoptosis**

Several vitamin D$_3$ analogs and 1,25(OH)$_2$D$_3$ have been shown to cause apoptosis of several type of cancer cells.$^{27,28}$ Gemini-19-nor and 1,25(OH)$_2$D$_3$ (10$^{-8}$ M, 4 days) induced 20% and 6% of HL-60 cells, respectively, to undergo apoptosis (Figure 6). At a higher concentration (10$^{-7}$ M, 4 days), both compounds produced apoptosis of about 20% of HL-60 cells (Figure 6).

**Analysis of the cell cycle and expression of p27$_{kip1}$**

The effect of Gemini-19-nor and 1,25(OH)$_2$D$_3$ on the cell cycle of the HL-60 cells was determined. A significant accumulation ($P < .05$) of cells in the G$_1$/G$_0$ and G$_2$/M phases of the cell cycle occurred, with a concomitant decrease in the proportion of those in S phase after 4 days of culture with either 1,25(OH)$_2$D$_3$ (10$^{-7}$ M) or Gemini-19-nor (10$^{-8}$ and 10$^{-7}$ M) (Figure 7).

The cyclin-dependent kinase inhibitor, p27$_{kip1}$, may act as a key regulator of G$_1$/G$_0$ accumulation induced by vitamin D$_3$. Both 1,25(OH)$_2$D$_3$ and Gemini-19-nor induced expression of p27$_{kip1}$ in a
dose-dependent manner at 4 days of exposure of HL-60 cells as determined by Western blot analysis (Figure 8A). Consistent with the cell cycle results, Gemini-19-nor strongly induced expression of p27kip1 at a lower concentration (10^{-9} M) than 1,25(OH)_{2}D_{3} (10^{-7} M). A time course study showed that Gemini-19-nor enhanced expression of p27kip1 by 6-fold at 0.5 days and about 36-fold at 3 days of exposure (Figure 8B).

We also examined if other inducers of differentiation of HL-60 cells could up-regulate expression of PTEN. The phorbol diester, TPA, induces macrophage-like differentiation and ATRA induces granulocyte-like differentiation of HL-60 cells. Four days of culture with either TPA (10^{-9} M) or ATRA (10^{-7} M) induced PTEN expression in HL-60 cells (Figure 8C).

**Discussion**

A recent study revealed that a vitamin D_{3} compound that has 2 side chains on C-20 (Gemini) was more active than 1,25(OH)_{2}D_{3} in its ability to inhibit clonal growth of malignant cells. Therefore, we synthesized additional novel Gemini compounds and examined their biologic effects on cancer cells. In this study, we evaluated 4 compounds from the newly synthesized family of Gemini. The Gemini-19-nor, which has 2 side chains on C-20 and the removal of the C-19, was the most potent inhibitor of clonal proliferation of myeloid leukemia, breast, and prostate cancer cells. It was more active than 1,25(OH)_{2}D_{3} in these 3 types of cancers. In particular, the analog showed marked activity with the NB4 acute promyelocyte leukemia cells and was 40 625-fold more potent than 1,25(OH)_{2}D_{3}. Therefore, we focused on the activity of this analog compared with 1,25(OH)_{2}D_{3}.

Previously, we reported that analogs of 1,25(OH)_{2}D_{3} that had removal of their C-19 moiety (19-nor 1,25D_{3} analogs) were active against prostate, breast, and myeloid leukemia cells. The 19-nor analog with the code name LH (1,25[OH]_{2}-16-ene-23-yne-26,27-F6-19-nor-D_{3}) was most potent against cancer cells of breast
markers, CD11b and CD14. Gemini-19-nor (10^{-8} M) induced expression of CD11b and CD14 in about 90% and 75% of cells, whereas the same concentration of 1,25(OH)_{2}D_{3} induced expression on only 50% and 30% of cells, respectively. Similarly, Gemini-19-nor (10^{-8} M) induced 70% of HL-60 cells to become NBT^{+}, compared to only 30% for the same concentration of 1,25(OH)_{2}D_{3}. Therefore, these results suggested that Gemini-19-nor was more potent than 1,25(OH)_{2}D_{3} as an inducer of myeloid differentiation.

Gemini-19-nor also mediated apoptosis. Previously, we reported that vitamin D_{3} analogs in concert with a RXR ligand induced apoptosis of myeloid leukemia cells and caused levels of expression of Bcl-2 to decrease suggesting an association between the 2 events.\textsuperscript{3,4,35} However, other experiments found that a vitamin D_{3} analog could induce apoptosis of an HL-60 variant without a reduction of cellular levels of Bcl-2.\textsuperscript{4} Another group reported that vitamin D_{3} compounds induced apoptosis via a novel caspase- and p53-independent pathway, and apoptosis was inhibited by forced expression of Bcl-2.\textsuperscript{36} These findings suggested that vitamin D_{3} might use several pathways to induce apoptosis.

Previous studies showed that vitamin D_{3} analogs caused accumulation at the G_{1}/G_{0} phase of the cell cycle, and this block may be mediated by p21^{waf1} and p27^{kip1} CDKIs.\textsuperscript{2,26,37} Gemini-19-nor D_{3} also produced G_{1}- to S-phase block of the cell cycle and induced p27^{kip1} expression. These results support the hypothesis that CDKIs mediated at least in part the antiproliferative effects of the vitamin D_{3} compounds by induction of a G_{1}/G_{0} accumulation. A block in the G_{2}/M checkpoint has also been previously observed in HL-60 cells treated with 1,25(OH)_{2}D_{3},\textsuperscript{38} and Gemini-19-nor was able to more potently induce a G_{2}/M block compared to 1,25(OH)_{2}D_{3}. This effect has been attributed to a decrease in levels of p34(cdc), a protein kinase which associates with B-type cyclins and controls transition through G_{2}/M. Therefore Gemini-19-nor may be able to decrease levels of this protein to a greater extent than 1,25(OH)_{2}D_{3}.

Figure 7. Cell cycle modulation by vitamin D_{3} compounds. HL-60 cells were cultured for 4 days with either 1,25(OH)_{2}D_{3} (10^{-7} M) or Gemini-19-nor (10^{-7} M), fixed, and stained with PI, and the cell cycle status was analyzed using flow cytometry. Column indicates mean (± SD) of 3 independent experiments. *P < .05 as determined by Student t test difference compared with the control group.
Recent studies indicated that the candidate tumor suppressor, PTEN, could block the phosphatidylinositol 3'-kinase (PI3K)/Akt signaling pathway, resulting in cell death or inhibition of growth or both.\(^2\) Active Akt mediates cell survival by inhibition of mitochondrial release of cytochrome c, inactivation of Forkhead transcription factors (FKHR), and phosphorylation of BAD and caspase-9.\(^4\) The overexpression of exogenous PTEN induced apoptosis of malignant cells.\(^5\)-\(^9\) Moreover, a genetic link between the Fas proapoptotic pathway and PTEN was suggested, because Fas-mediated apoptosis was impaired in the germline heterozygous PTEN\(^10\) murine model.\(^11\) In addition, PTEN induced G1 cell cycle arrest and this was associated with an increased expression of p27\(^kip1\).\(^12\)-\(^15\) In the present study, Western blotting analysis demonstrated that PTEN expression was up-regulated by vitamin D\(_3\) compounds in HL-60 cells, and it paralleled the induction of apoptosis, expression of p27\(^kip1\) and G1 cell cycle arrest. These findings suggest that PTEN might enhance apoptosis and G1 cell cycle arrest in transformed cells exposed to a vitamin D\(_3\) analog, and it might help explain the multiple pathways of apoptosis mediated by vitamin D\(_3\) compounds.

Several studies indicated that PTEN induced cell differentiation in glioma cells.\(^16\)-\(^18\) Therefore, we analyzed inducers of myeloid differentiation other than vitamin D\(_3\). We choose TPA, a stimulator of the protein kinase C pathway that induces macrophage-like differentiation, and ATRA, which binds the nuclear hormone receptor, retinoic acid receptor and induces granulocytic differentiation. As shown in Figure 6C, TPA and ATRA significantly induced PTEN expression. These observations suggest that PTEN expression is associated with monocytic and granulocytic differentiation. We do not know if this marked increase in PTEN expression is the cause or the effect of terminal differentiation of HL-60 cells. Further studies are required to define the role PTEN plays in this process of myeloid differentiation.

Taken together, the new vitamin D\(_3\) analog Gemini-19-nor D\(_3\) strongly inhibited growth of transformed cells, and produced myeloid differentiation, apoptosis, and G1/G0 arrest associated with elevated levels of p27\(^kip1\). Moreover, the vitamin D\(_3\) analog induced expression of PTEN. These observations suggest that the anticancer effects of vitamin D\(_3\) might be regulated in part via PTEN. This analog may provide an adjuvant therapy for myeloid leukemia, especially acute promyelocytic leukemia, and may be effective in other types of cancers.

![Figure 8. Induction of expression of p27\(^kip1\) and PTEN by vitamin D\(_3\) compounds.](image)

(A) Dose-dependent study of p27\(^kip1\) and PTEN expression in HL-60 cells analyzed by Western blot. Cells were either untreated (control) or cultured with 10\(^{-5}\) to 10\(^{-7}\) M of either 1,25(OH)\(_2\)D\(_3\), or Gemini-19-nor for 4 days. GAPDH was used as a loading control. (B) Time course study of p27\(^kip1\) and PTEN expression in HL-60 cells studied by Western blot. Cells were either untreated (Control) or cultured with Gemini-19-nor (10\(^{-7}\) M) for 0.5 to 4 days. GAPDH was used as a loading control. (C) Induction of PTEN expression by TPA and ATRA in HL-60 cells. Cells were either untreated (control) or cultured with either TPA (10\(^{-7}\) M) or ATRA (10\(^{-7}\) M) for 4 days. GAPDH was analyzed as a loading control.

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