1α,25-Dihydroxy-20-Epi-Vitamin D₃: An Extraordinarily Potent Inhibitor of Leukemic Cell Growth In Vitro

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We have evaluated seven recently synthesized vitamin D₃ analogs for their abilities to inhibit clonal growth of leukemic cells, to induce leukemic cell differentiation, to stimulate clonal growth of normal myeloid committed stem cells, and to transactivate a reporter gene having a 1,25(OH)₂D₃ response element (VDRE). The 1,25(OH)₂-20-epi-D₃ showed extraordinary activity; at 10⁻¹¹ mol/L it inhibited clonal growth of 87% of HL-60 myeloblast cells, 60% of S-LB1 cells (human T-cell lymphotropic virus type 1 [HTLV-1]-immortalized human T-lymphocyte cell line) and 50% of leukemic clonogenic cells (colony-forming unit-leukemia) obtained from patients with acute myelogenous leukemia. No effect of either 1,25(OH)₂D₃ or 1,25(OH)₂-20-epi-D₃ was observed on the clonal proliferation of an HTLV-1-immortalized human T-lymphocyte cell line (Ab-VDR) having nonfunctional 1,25(OH)₂D₃ cellular receptors (VDR). The abilities of 1,25(OH)₂-20-epi-D₃ to induce differentiation of HL-60 cells, as measured by generation of superoxide and nonspecific esterase production, was less than its antiproliferative activities. This analog stimulated colony-forming unit-granulocyte-macrophage growth from normal human bone marrow. To gain insights into the remarkable antileukemic activities of 1,25(OH)₂-20-epi-D₃, we examined its ability to enter HL-60 cells, bind to the VDR, and interact with a transfected VDRE attached upstream of a TK promoter-driven reporter gene (chloramphenicol acetyl transferase [CAT]). The 1,25(OH)₂-20-epi-D₃ potently increased CAT activity (>16-fold, as compared with cells transfected with control receptor having no VDRE); paradoxically, 1,25(OH)₂-20-epi-D₃ was of equal potency to 1,25(OH)₂D₃ in transactivating the VDRE-containing reporter gene, even though the analog had a 1,000-fold greater antileukemic effect as compared with 1,25(OH)₂D₃. In summary, we have identified an extremely potent 1,25(OH)₂D₃ analog with antiproliferative and differentiating effects on leukemic cells and that may be clinically useful. This analog appears to generate biologic responses via the classical VDR pathway, but further studies are required to elucidate the mechanism by which this analog produces its prominent activities.

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The seco-steroid hormone 1α-25-dihydroxyvitamin D₃ [1,25(OH)₂D₃, code name C] is responsible for calcium homeostasis in humans. Evidence is now convincing that 1,25(OH)₂D₃ interacts very closely with the hematopoietic system. (1) Monocytes, macrophages, activated lymphocytes, and leukemic cells have receptors for 1,25(OH)₂D₃. (2) 1,25(OH)₂D₃ stimulates proliferation and modulates differentiation of the normal myeloid stem cells to monocytes/macrophages. (3) Low concentrations of 1,25(OH)₂D₃ inhibit the proliferation and induce neoplastic cells from several myeloid cell lines (M1, HL-60, and U-937) to differentiate to macrophage-like cells. (4) Activated normal macrophages can synthesize 1,25(OH)₂D₃, which belong to the steroid receptor superfamily. These receptors act as ligand-dependent transcription factors that bind to specific DNA sequences. Acute myelogenous leukemia (AML) arises from neoplastic transformation of a myeloid stem cell; these leukemic cells are unable to undergo cellular maturation at an early stage of development. High-dose chemotherapy has improved survival of AML patients, but severe bone marrow depression limits its use. An ideal alternative therapy for these patients is to induce differentiation and/or inhibit clonal proliferation of their leukemic cells without toxic effects on their normal hematopoietic stem cells. Studies in vivo suggest that 1,25(OH)₂D₃ is able to prolong the survival of mice injected with leukemic cells. A trial of oral administration of 1,25(OH)₂D₃ to preleukemic patients was partially effective, perhaps because concentrations required to see activity in vitro could not be achieved in vivo unless hypercalcemia developed. Therefore, research activities have been directed at finding new 1,25(OH)₂D₃ analogs with a more favorable therapeutic profile.

In the present study, we have analyzed a variety of 1,25(OH)₂D₃ analogs in vitro. The 1,25(OH)₂-20-epi-D₃ (code name IE and also known as MC1288) belongs to a new series of 1,25(OH)₂D₃ analogs characterized by an inversion of the stereochemistry at carbon 20 (C20). It showed extraordinary activities. Analog IE at 10⁻¹¹ mol/L inhibited clonal growth of greater than 85% HL-60 myeloblastic leukemia cells, 60% S-LB1 cells (human T-cell lymphotropic virus type 1 [HTLV-1]-immortalized human T-lymphocyte cell line from a normal individual) and 50% of leukemic myeloid clonogenic cells (colony-forming unit-leukemia [CFU-L]) from patients with AML. In contrast, analog IE stimulated growth of granulocyte-macrophage colony-forming units (CFU-GM) from normal human bone marrow.
VITAMIN D ANALOG: INHIBITOR OF LEUKEMIC CELLS

marrow. To gain insights into the remarkable antileukemic activities of analog IE, we examined its ability to inhibit the clonal growth of a hematopoietic cell line having no functional VDR and examined its ability to enter HL-60 cells and interact with a transfected 1,25(OH)2D3 response element (VDRE) attached upstream of a TK promoter-driven reporter gene (chloramphenicol acetyl transferase [CAT]).

MATERIALS AND METHODS

Cell. The following cell lines were used in this study: HL-60 cells are late promyeloblasts established from a patient with acute myeloid leukemia; S-LB1 is an HTLV-1-immortalized human T-lymphocyte line from a normal individual; and Ab-VDR is an HTLV-1-immortalized human T-lymphocyte cell line established from a patient with vitamin D-resistant rickets type II (these cells have undetectable 1,25(OH)2D3 cellular receptors). The cells were grown in tissue culture flasks in α-medium (Flow Laboratories, McLean, VA) with 10% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA) and 1% penicillin/streptomycin (Sigma Chemical Co, St Louis, MO).

Bone marrow for the CFU-GM study was obtained from 5 healthy volunteers by aspiration from the iliac crest. Peripheral blood for CFU-GM study was drawn from 4 patients with AML (M1, M2, M4, and M5a). Individuals from both groups provided informed consent. The percentage of circulating blast cells was more than 98%. Blood and bone marrow were anticoagulated by addition of preservative-free heparin (Sigma). Mononuclear cells (MNC) from normal bone marrow and peripheral blood were collected by separation on Ficoll-Paque (Pharmacia, Inc, Piscataway, NJ) gradients at a density of 1.077, washed twice in phosphate-buffered saline (PBS), and suspended in iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) containing 20% heat-inactivated fetal bovine serum (GIBCO).

Analogs of 1,25(OH)2D3. The eight analogs of 1,25(OH)2D3 used in this study are shown in Fig 1: their two-letter code name is indicated. The synthetic steroid hormone [1,25(OH)2D3], cmpd C was provided by Dr Milan Uskokovic of the Hoffmann LaRoche Co (Nutley, NJ); analog IE, also known as MC1288, was synthesized at LEO Pharmaceutical Products and was provided by L. Binderup; and analogs HF, HH, HI, HJ, HO, and HR were provided by Drs W.H. Okamura, K.R. Murakami, R.L. Craig, and M. Curtin at the University of California at Riverside. All analogs were dissolved in absolute ethanol at 10-3 mol/L as stock solutions; these were stored at -20°C and protected from light. Immediately before use, stock solutions were diluted in α-medium. The final alcohol concentration in the cultures did not exceed 0.1% and this concentration did not influence either cellular growth or differentiation (data not shown).

Studies of induction of differentiation. Nitroblue (NBT) reduction was assayed as described previously. α-Naphthyl acetate esterase activity (NAAE) was examined on cytospin preparations using a Sigma kit (Sigma). The cells were grown in liquid culture with α-medium, 10% FCS for 6 days in a humidified atmosphere, 5% CO2 at 37°C with and without vitamin D3 analogs.

 Colony formation assay in soft-gel. Human cell lines and normal bone marrow cells (NBMC) were cultured in a two-layer soft agar system as previously described. The culture medium was α-medium for cell lines and IMDM for NBMC, each with 20% FCS. For CFU-GM of NBMC, we added 5 x 10-2 mol/L β-mercaptoethanol (Sigma). As a source of CSF for CFU-GM, 200 pmol/L of GM-CSF was used (generous gift from S. Clark, Genetics Institute, Boston, MA). The circulating blast cells from 4 AML patients were cultured in IMDM, 20% FCS, 10% phytohemagglutinin–lyeocyte-conditioned medium (PHA-LCM) and 10-4 mol/L β-mercaptoetha-nol in a final concentration of 0.8% methylcellulose (Mithocel MC 4000 CP; Fluka AG, Buchs, Switzerland). All cells were plated in 6-well culture plates (Becton Dickinson Labware, NJ) containing different concentrations of vitamin D compounds. Cell concentrations were 2 x 105 per plate for leukemia cell lines and 1 x 106 for NBMC and AML blasts. After 10 days of incubation at 37°C in a humidified atmosphere containing 5% CO2 in air, colonies (≥20 cells for HL-60, S-LB1, and AB-VDR cell lines and ≥40 cells for fresh AML samples) were counted using an inverted microscope. All experiments were performed using triplicate plates per experimental point.

 Transfection and assay of CAT activity. HL-60 cells were grown in α-medium with 5% FCS and 1% penicillin/streptomycin. After washing in serum-free medium, 2.5 x 105 cells were transfected by electroporation with 35 μg of pBL-CAT2-VDRE plasmid (synthesized complementary sequences comprising human osteocalcin gene between -509 and -489 [3 repeats] fused to pBL-CAT2 expression vector containing thymidine kinase promoter). The cells were cultured with IE [1,25(OH)2-20-epi-D3] or C [1,25(OH)2] at 10-10 to 10-7 mol/L for 48 hours in serum-free conditions (Serumless Medium; GIBCO) and harvested, and CAT lysates were prepared. CAT activity was assayed by thin-layer chromatography and autoradiography.

RESULTS

Effects of 1,25(OH)2D3 analogs on clonal growth of HL-60 cells. We examined the effect of 1,25(OH)2D3 (C) and its analogs on clonal proliferation of the myeloid leukemic cell line HL-60 (Fig 2). Concentrations that inhibited 50% growth (ED50) are shown in Table 1. Four analogs (IE, HQ, HR, and HF) as well as C [1,25(OH)2D3] inhibited clonal growth of leukemic HL-60 cells in a dose-dependent fashion; the remaining three analogs (HH, HI, and HL) had little effect on the clonal growth of HL-60 cells. The reference compound, 1,25(OH)2D3 (C), achieved a 50% inhibition of clonal growth at 1.6 x 10-8 mol/L. The most potent analog was IE [1,25(OH)2-20-epi-D3], having an ED50 of about 6 x 10-10 mol/L (1% colony formation at 1 x 10-11 mol/L). Therefore, an alteration of stereochemistry at carbon 20 increased potency more than 2,500-fold. Although not as effective as the side chain analog IE in inhibiting clonal growth, the analogs HR and HQ, which possess diastereomeric allenic functions in their side chains, are slightly more effective than the natural hormone 1,25(OH)2D3 (C). By contrast, stereochemical alterations of hormone C at carbon 1 (HL), carbon 3 (HJ), or at both carbons 1 and 3 (HH) significantly decreased potency. Surprisingly, HF, an analog with completely different AB ring topology (resembling a steroid-like conformation rather than the extended conformation of IE, C, and the other analogs depicted in Fig 1), still exhibited some ability to inhibit clonal growth. The rank order of vitamin D3 analogs from most to least potent in terms of clonal growth was IE > > > HR > HQ > C > HF > HL = HJ > HH.

Effect of 1,25(OH)2D3 analogs on differentiation of HL-60 cells. The HL-60 cells are able to differentiate toward monocytes/macrophages when cultured in the presence of 1,25(OH)2D3. One of the markers of this cellular differentiation is the ability to produce superoxide as measured by the capacity of the cells to reduce NBT. We examined the ability of 1,25(OH)2D3 analogs to reduce NBT in HL-60
cells (Table 1 and Fig 3). The most potent inducer of differentiation was analog IE, with an ED$_{50}$ of $5.8 \times 10^{-9}$ mol/L. The rank order of potencies of induction of differentiation of vitamin D$_3$ compounds was similar to their rank order of activities in inhibiting proliferation of the HL-60 cells. An additional marker of monocyte-macrophage differentiation is the acquisition of NAE, an enzyme that is detected in monocytes and macrophages, but is absent in more immature myeloid blast cells. Dose-response studies were performed for 1,25(OH)$_2$-20-epi-D$_3$ and 1,25(OH)$_2$D$_3$ (data not shown) and the ED$_{50}$ values were calculated (Table 1). The ED$_{50}$ for development of expression of NAE was $1.2 \times 10^{-9}$ mol/L.
Table 1. Effect of Vitamin D$_3$ Analogs on Cellular Proliferation and Differentiation of HL-60 Cells

<table>
<thead>
<tr>
<th>Chemical Name of Analogs</th>
<th>Brief Name</th>
<th>Inhibition of Clonal Growth</th>
<th>NBT</th>
<th>NAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α,25-(OH)$_2$D$_3$</td>
<td>C</td>
<td>16</td>
<td>37</td>
<td>70</td>
</tr>
<tr>
<td>1α,25-(OH)$_2$-20-epi-D$_3$</td>
<td>IE</td>
<td>0.006</td>
<td>5.8</td>
<td>1.2</td>
</tr>
<tr>
<td>(22S)-1,25-(OH)$_2$-22,23-diene-D$_3$</td>
<td>HQ</td>
<td>11</td>
<td>60</td>
<td>ND</td>
</tr>
<tr>
<td>(22R)-1,25-(OH)$_2$-22,23-diene-D$_3$</td>
<td>HR</td>
<td>5.2</td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25-(OH)$_2$-pre-D$_3$-9,19,19,19-d$_3$</td>
<td>HF</td>
<td>22</td>
<td>86</td>
<td>ND</td>
</tr>
<tr>
<td>1β,25-(OH)$_2$-3-epi-D$_3$</td>
<td>HH</td>
<td>NR</td>
<td>NR</td>
<td>ND</td>
</tr>
<tr>
<td>1α,25-(OH)$_2$-3-epi-D$_3$</td>
<td>JH</td>
<td>NR</td>
<td>NR</td>
<td>ND</td>
</tr>
<tr>
<td>1β,25-(OH)$_2$D$_3$</td>
<td>HL</td>
<td>NR</td>
<td>NR</td>
<td>ND</td>
</tr>
</tbody>
</table>

Standard deviations were less than 10%.
Abbreviations: NR, ED$_{50}$ was not reached even at 10$^{-7}$ mol/L; ND, none done.

Effect of vitamin D$_3$ analogs on clonal proliferation of T-lymphocyte cell lines S-LB1 and Ab-VDR. We have used HTLV-I-immortalized T lymphocytes from a normal individual (S-LB1) as well as from a patient with vitamin D-resistant rickets type II (Ab-VDR). Ab-VDR has nonfunctional 1,25(OH)$_2$D$_3$ nuclear receptors. The 1,25(OH)$_2$-20-epi-D$_3$ had a strong inhibitory effect on clonogenic growth of S-LB1 cell line, with an ED$_{50}$ of 3.5 × 10$^{-11}$ mol/L (Fig 4). The 1,25(OH)$_2$D$_3$ slightly stimulated the clonal growth of S-LB1 cells at 10$^{-11}$ to 10$^{-10}$ mol/L and inhibit the clonal proliferation at 10$^{-9}$ to 10$^{-7}$ mol/L, with an ED$_{50}$ of 6 × 10$^{-9}$ mol/L. Neither 1,25(OH)$_2$-20-epi-D$_3$ nor 1,25(OH)$_2$D$_3$ (10$^{-11}$ to 10$^{-7}$ mol/L) affected growth of Ab-VDR cells (Fig 4).

Effect of 1,25(OH)$_2$-20-epi-D$_3$ and 1,25(OH)$_2$D$_3$ on clonogenic proliferation of normal and leukemic human myeloid clonaligenic cells. The normal committed myeloid stem cells, CFU-GM, were stimulated in their clonal growth by 1,25(OH)$_2$D$_3$ (C) and 1,25(OH)$_2$-20-epi-D$_3$ (IE) at 10$^{-10}$ to 10$^{-8}$ mol/L, with peak enhancement at 10$^{-8}$ mol/L. At 10$^{-7}$ mol/L, both compounds inhibited clonal growth of CFU-GM. Further experiments examined the effects of 1,25(OH)$_2$D$_3$ (C) and 1,25(OH)$_2$-20-epi-D$_3$ (IE) on the clonal growth of leukemic cells from 4 individuals with acute myeloid leukemia (CFU-L). Both compounds (10$^{-11}$ to 10$^{-7}$ mol/L) inhibited the proliferation of CFU-L with a 50% inhibition of clonal growth at about 10$^{-10}$ mol/L for 1,25(OH)$_2$-20-epi-D$_3$ (IE) and 5 × 10$^{-8}$ mol/L for 1,25(OH)$_2$D$_3$ (C).

Effect of 1,25(OH)$_2$-20-epi-D$_3$ and 1,25(OH)$_2$D$_3$ on transcriptional activation. The 1,25(OH)$_2$D$_3$ response element (VDRE) was placed in front of a thymidine kinase promoter of the reporter gene CAT. This construct was transfected into HL-60 cells. In the absence of 1,25(OH)$_2$D$_3$ (C) or 1,25(OH)$_2$-20-epi-D$_3$, almost no CAT activity was detectable (Fig 6A and B). Both 1,25(OH)$_2$D$_3$ (C) and 1,25(OH)$_2$-20-epi-D$_3$ increased CAT activity in a dose-response manner. At 10$^{-7}$ mol/L, both compounds increased CAT activity nearly 16-fold as compared with cells transfected with the reported vector in the absence of vitamin D$_3$ compounds (Fig 6A and B).

DISCUSSION
The 1,25(OH)$_2$-20-epi-D$_3$ (analog IE) belongs to the new group of 1,25(OH)$_2$D$_3$ analogs, characterized by an inverted stereochemistry at carbon 20 of the side-chain. A previous
The 20-epi analogs of 1,25(OH)2D3 analogs were shown to inhibit cytokine-mediated T-lymphocyte activation.26 The cell line S-LB1 was established by HTLV-1 infection of T lymphocytes from a normal individual.28 We previously showed that the 1,25(OH)2D3 nuclear receptor in this cell line behaves in a comparable fashion as the 1,25(OH)2D3 receptor in intestinal cells.42 In these studies, we have observed that the 1,25(OH)2-20-epi-D3 (IE) was 100-fold more potent than 1,25(OH)2D3 (C) in inhibition of clonal growth of the S-LB1 cell line. The 1,25(OH)2-20-epi-D3 probably deserves further investigation as an immunosuppressive agent.

The alteration of stereochemistry at carbon 20 on the side-chain is the only difference between 1,25(OH)2D3 and 1,25(OH)2-20-epi-D3. The 8 carbon side-chain with a 20R orientation (the orientation of the natural hormone) is unusually flexible and can attain a wide population of orientations; it is not yet known which side-chain conformation(s) represents that of the ligand bound to the VDR. Inversion of the stereochemistry at carbon-20 (from 20R to 20S, as in analog IE) allows the side-chain to access the appropriate side-chain hydroxyl topology; ie, IE's side-chain may be more optimally preorganized (thus lowering its conformational entropy) into the biologically competent orientation. Future studies need to test this hypothesis.

Although side-chain analog IE was the most potent analog in the inhibition of clonal growth, the analogs HR and HQ, which possess diastereomeric allenic functions in the side-chain, are slightly more effective than the natural hormone analogs.
1,25(OH)_{2}D_{3} (C). By contrast, stereochemical alterations of hormone C at carbon 1 and/or either carbon 3 by epimerizing one of the hydroxyls (HL, HJ, or HH) displayed little biologic activity. However, less explicable is the finding that HF, an analog with a completely different A and B ring topology (resembling a steroid-like conformation rather than the extended conformation of IE, C, and the other analogs reported here), exhibited a significant ability to inhibit clonal growth. Perhaps under the assay conditions, analog HF isomerizes to structure C, a known isomerization process.\(^{31}\) Overall, the stereochemical topology of the A-ring hydroxyls appears to have an extraordinary effect at least on the inhibition of clonal growth (Fig 2) as well as in eliciting cellular differentiation properties (Fig 3).

Recently, evidence has accumulated that some effects of 1,25(OH)_{2}D_{3} analogs may be mediated independent of 1,25(OH)_{2}D_{3} nuclear receptors. For example, several 1,25(OH)_{2}D_{3} analogs have been found to mediate intracellular Ca\(^{2+}\) fluxes through a nongenomic mechanism that is independent of the classical pathway of receptor mediated activity.\(^{29}\) In this system, the analog HL [1\(\beta,25(OH)_{2}D_{3}\)] was found to be a stereoselective antagonist of the nongenomic actions of 1,25(OH)_{2}D_{3}, but without effect on the nuclear receptor mediated regulations of gene transcripts.\(^{2}\)

One series of experiments suggests that 1,25(OH)_{2}20-epi-D_{3} mediates its effects through the vitamin D\(_{3}\) receptor. We established a T-cell line (Ab-VDR) from a patient with vitamin D-dependent rickets type II, by infecting the lymphocytes of the patient with HTLV-1.\(^{28}\) These cells do not have functional vitamin D\(_{3}\) receptors. Clonal growth of these cells was not altered in the presence of high concentrations (10^{-7} mol/L) of either 1,25(OH)_{2}20-epi-D_{3} (IE) or 1,25(OH)_{2}D_{3} (C) (Fig 4). In contrast, the matched control, S-LBl cells (HTLV-1-transformed T cells from a normal individual) having functional vitamin D\(_{3}\) receptors, were markedly inhibited by 1,25(OH)_{2}20-epi-D_{3} (ED_{50}, 3 \times 10^{-11} mol/L).
This result would suggest that 1,25(OH)2-20-epi-D3 is not mediating its effects through a non–1,25(OH)2D3 receptor mechanism.

Potentially, 1,25(OH)2-20-epi-D3 (IE) might have either higher affinity for the 1,25(OH)2D3 nuclear receptors or the ligand/receptor complex might interact more efficiently with the VDRE to modulate gene expression. To pursue these possibilities, a reporter gene containing the VDRE was transfected into HL-60 cells. The cells were cultured in media containing different concentrations of either 1,25(OH)2-20-epi-D3 (IE) or 1,25(OH)2D3 (C) and reporter gene activities were measured. These two vitamin D3 compounds had nearly identical activities (Fig 6), strongly suggesting that the difference between 1,25(OH)2-20-epi-D3 (IE) and 1,25(OH)2D3 (C) cannot be ascribed to differential abilities either to enter the cells, or to bind to VDR and then to VDRE and transactivate a target gene. Potentially, the 1,25(OH)2-20-epi-D3/VDR complex may interact with a different group of VDREs than those bound by the 1,25(OH)2D3/VDR complex; the testing of this possibility will have to await the identification of appropriate target genes that control myeloid differentiation.

In the current study, we have shown that 1,25(OH)2-20-epi-D3 is among the most potent 1,25(OH)2D3 analogs as a modulator of induction of differentiation and inhibition of clonal proliferation of leukemic cells without inhibition of normal myeloid clonal growth. Further in vivo studies of this compound are needed; this novel vitamin D3 analog or members of this family may eventually have therapeutic potential.

REFERENCES

22. Evans RM: The steroid and thyroid hormone receptor superfamily. Science 240:889, 1988
30. Craig AS, Norma AW, Okamura WH: Two novel allenic


1 alpha,25-Dihydroxy-20-epi-vitamin D3: an extraordinarily potent inhibitor of leukemic cell growth in vitro

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