Novel Vitamin D Analogs That Modulate Leukemic Cell Growth and Differentiation With Little Effect on Either Intestinal Calcium Absorption or Bone Calcium Mobilization


Induction of terminal differentiation of leukemic and preleukemic cells is a therapeutic approach to leukemia and preleukemia. The 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the hormonally active form of vitamin D₃, can induce differentiation and inhibit proliferation of leukemia cells, but concentrations required to achieve these effects cause life-threatening hypercalcemia. Seven new analogs of 1,25(OH)₂D₃ were discovered to be either equivalent or more potent than 1,25(OH)₂D₃ as assessed by: (a) inhibition of clonal proliferation of HL-60, EM-2, U937, and patients' myeloid leukemic cells; and (b) induction of differentiation of HL-60 promyelocytes. Furthermore, these analogs stimulated clonal growth of normal human myeloid stem cells. The most potent analog, 1,25-dihydroxy-16ene-23yne vitaminD₃, was about fourfold more potent than 1,25(OH)₂D₃. This analog decreased clonal growth and expression of c-myc oncogenes in HL-60 cells by 50% within ten hours of exposure. Effects on calcium metabolism of these novel analogs in vivo was assessed by intestinal calcium absorption (ICA) and bone calcium mobilization (BCM). Each of the analogs mediated markedly less (10 to 200-fold) ICA and BCM as compared with 1,25(OH)₂D₃. To gain insight into the possible mechanism of action of these new analogs, receptor binding studies were done with 1,25(OH)₂D₃,16ene-23yne-D₃, and showed that it competed only about 60% as effectively as 1,25(OH)₂D₃ for 1,25(OH)₂D₃ receptors present in HL-60 cells and 98% as effective as 1,25(OH)₂D₃ for receptors present in chick intestinal cells. In summary, we have discovered seven novel vitamin D analogs that are more potent than the physiologic 1,25(OH)₂D₃ as measured by a variety of hematopoietic assays. In contrast, these compounds appear to have the potential to be markedly less toxic (induction of hypercalcemia). These novel vitamin D compounds may be superior to 1,25(OH)₂D₃ in a number of clinical situations including leukemia/preleukemia; they will provide a tool to dissect the mechanism of action of vitamin D seco-steroids in promoting cellular differentiation.

A PHENOTYPIC abnormality of acute leukemia is their inability to differentiate into functional, mature cells.1 Instead, they remain in the proliferative pool providing them a distinct growth advantage over their normal counterpart. Patients with acute leukemias usually die of either infection or hemorrhage because of neutropenia and thrombocytopenia. One possible approach to treatment of patients with acute leukemia is to induce differentiation and/or to inhibit clonal proliferation of their leukemic cells, thereby eliminating these cells from the proliferative pool.

Several compounds induce acute myelogenous leukemia (AML) cells to differentiate. One of these is 1α,25 dihydroxyvitamin D₃ [1,25(OH)₂D₃], which is the hormonally active metabolite of vitamin D₃ that mediates calcium absorption from gut and reabsorption from bone.2,4 The treatment costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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Submitted December 30, 1988; accepted February 28, 1989.

Supported in part by Public Health Service Grants No. CA 26038, CA 32737, CA 33936, and CA30512 and the 4E Leukemia Fund in memory of Marilyn Levine, Erwin Epstein, Harold Blass, Goldie Berman and in honor of Roselle Lewis.

P.K. is a member of the Jonsson Comprehensive Cancer Center, and has a Career Development Award from the National Institutes of Health.

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METHODS

Cells. Leukemic cell lines used in this study are as follows: KG-1, early myeloblasts established from a patient with erythroleukemia;3 EM-2, myeloblasts from a patient with myeloblast crisis of chronic myelogenous leukemia (CML);4 HL-60, promyelocytes from a patient with acute myeloid leukemia5; U937, monoblasts from a patient with acute monoblastic leukemia;6 BV173, lymphocytes from a patient with lymphoid blast crisis of CML.7 The cells
were cultured in tissue culture flasks (Lux Miles Laboratories, Inc, Naperville, IL) in alpha medium (Flow Laboratories, Inc, McLean, VA) with 10% fetal calf serum (FCS; Irvine Scientific, Santa Ana, CA). Only cells in logarithmic growth were used for plating experiments.

Bone marrow was obtained from healthy volunteers and leukemic patients by aspiration after written consent was obtained. The mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradients, washed twice in 1× phosphate-buffered saline (PBS), and suspended in alpha medium containing 10% FCS. Peripheral blood mononuclear cells were obtained using similar techniques from consenting leukemia patients who had a high percentage (>85%) of leukemic cells in their blood.

Vitamin D compounds. The vitamin D compounds used in these experiments are listed in Fig 1 and were synthesized by Dr M. Uskokovic at Hoffman-LaRoche. Each was dissolved in absolute ethanol at 10⁻³ mol/L to create a stock solution; this was stored at −20°C and protected from light. Dilutions of the stock solution were made in alpha medium without FCS. The maximum concentration of ethanol in the culture (0.1%) did not influence cell growth or differentiation.

Colonies formed in soft agar and analysis of differentiation. Cells were plated in Lux culture dishes in a two-layer soft agar system according to previously described methods. Recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF) (200 p-mol/L, maximally stimulating concentration, data not shown) was used as a source of CSF for KG-1 and normal marrow cells (S. Clark, Genetics Institute, Boston). Cell concentrations were 2 × 10³/plate for leukemic cell lines and 2 × 10⁵/plate for normal bone marrow and leukemic cells from patients. All experiments contained three dishes per experimental point using control plates with either no CSF or only CSF. Each experiment was performed at least twice. Colonies (≥40 cells) were scored with an inverted microscope.
Induction of differentiation was measured both by reduction of nitroblue tetrazolium (NBT) and by histochemistry using α-naphthyl acetate esterase (NSE). The cells were grown in liquid culture with alpha medium, 10% FCS for six days in humidified 7.0% CO₂ atmosphere at 37°C. For NBT, cell suspension (2 x 10⁶ cells/mL) was mixed with an equal volume of solution containing 1.25 mg/mL NBT (Sigma Chemical Co, St Louis), 17 mg/mL bovine serum albumin and 1 μg/mL 12-0-tetradecanoylphorbol 13-acetate (Miles Laboratories) for 30 minutes at 37°C. The cells were washed in PBS, cytocentrifuged, fixed in methanol for five minutes, and stained with gram safranin for ten minutes. For NSE, slides were fixed for 30 seconds at room temperature in citrate-acetone-formaldehyde solution and were stained with mixed solution of 1 mL fast blue BB base solution and 1 mL sodium nitrate solution, 40 mL prewarmed deionized water, 5 mL trizmal, pH 7.6, and 1 mL naphthyl acetate solution for 30 minutes at 37°C, protected from light.

RNA isolation and blotting. Total RNAs of HL-60, EM-2, KG-1 cells were extracted by the hot phenol method. RNA blotting was basically performed as described by Maniatis et al. Blots were probed with [³²P] oligolabeled restriction fragments. Filters were washed to a stringency of 0.1 x SSC at 65°C and exposed to Kodak XAR film overnight at -70°C. Autoradiograms were developed at different exposures and scanned by densitometry. Probes used were the second exon (PstI-PstI fragment) of c-myc and the 3' nontranslated region of β-actin.

Assays of intestinal calcium absorption and bone calcium mobilization. Intestinal calcium absorption (ICA) and bone calcium mobilization (BCM) were measured in vivo, as described previously. Twelve hours before assay, the chickens, which had been placed on a zero-calcium diet 48 hours before assay, were injected intramuscularly with the vitamin metabolite or analog dissolved in 0.2 mL ethanol:1,2-propanediol (1:1, vol/vol) or with vehicle. At the time of assay, 4.0 mg ⁴⁰Ca⁺⁺ + 5 μCi of ⁴⁰Ca⁺⁺ (New England Nuclear) were placed in the duodenum of the birds lightly anesthetized with ether. After 30 minutes, the birds were decapitated and the blood was collected. The radioactivity content of 0.2 mL serum was measured in a liquid scintillation counter (Beckman LS8000) to

Fig 2. Dose-response of vitamin D analogs on clonal proliferation of leukemic cells from myeloid lines. (A) Effect of vitamin D analogs on clonal proliferation of HL-60 promyelocytes. (B) Effect of vitamin D analogs on EM-2 myeloblasts. (C) Effect of vitamin D analogs on U937 monoblasts. Results are expressed as a percent of control plates not exposed to vitamin D compounds (control cultures contain a mean 195 colonies ± 10 SE for HL-60; mean 186 ± 39 SE for EM2; mean 218 ± 36 SE for U937). Each point represents mean of two experiments with triplicate dishes (△). 1,25-(OH)₂D₃; (○). 1,25-(OH)₂-16ene-D₃; (△). 1,25S,26-(OH)₃D₂; (□). 1,25R,26-(OH)₃-22ene-D₃; (X). 1,25,28-(OH)₃D₂.
Table 1. Effect of Vitamin D Analogs on Leukemic Clonal Proliferation and Cellular Differentiation

<table>
<thead>
<tr>
<th>No.</th>
<th>Analog</th>
<th>Colony Formation</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ED₅₀ (x 10⁻⁹ mol/L)</td>
<td>NBT</td>
</tr>
<tr>
<td>1</td>
<td>1,25-(OH)₂-D₃</td>
<td>16</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>1,25-(OH)₂-16ene-23yne-D₃</td>
<td>29</td>
<td>30</td>
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<td>3</td>
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<td>6</td>
</tr>
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<td>4</td>
<td>1,25S,26-(OH)₁-D₃</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>1,25R,26-(OH)₁-22ene-D₃</td>
<td>8</td>
<td>31</td>
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<tr>
<td>6</td>
<td>1,25S,26-(OH)₁-22ene-D₃</td>
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<td>7</td>
<td>1,23S,25-(OH)₁-D₃</td>
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<td>49</td>
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<td>8</td>
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<td>23</td>
<td>76</td>
</tr>
<tr>
<td>9</td>
<td>1,25,28-(OH)₁-D₃</td>
<td>NR</td>
<td>49</td>
</tr>
</tbody>
</table>

*Abbreviations: ED₅₀, effective dose achieving 50% response; NBT, nitroblue tetrazolium; NSE, nonspecific esterase; NR, ED₅₀ was not reached.*

Fig 3. Dose-response of vitamin D analogs on clonal proliferation of leukemic cell lines and normal human marrow cells. (A) Effect of vitamin D analogs on KG-1 early myeloblasts. (B) Effect of vitamin D analogs on BV-173 lymphoblasts. (C) Effect of vitamin D analogs on normal human myeloid colony-forming cells. Each point represents the mean of two experiments with triplicate dishes. Results are expressed as percent of control cells not exposed to vitamin D compounds (control cultures contain mean 49 colonies ± 9 SE for KG-1; mean 268 colonies ± 69 SE for BV173; mean 85 colonies ± 12 SE for normal human GM-CFC). (△), 1,25-(OH)₂-D₃; (□), 1,25-(OH)₂-16ene-23yne-D₃; (○), 1,25-(OH)₂-22yne-D₃; (●), 1,25S,26-(OH)₁-D₃; (◇), 1,25R,26-(OH)₁-22ene-D₃; (X), 1,25S,26-(OH)₁-22ene-D₃; (●), 1,23S,25-(OH)₁-D₃; (○), 1,23R,25-(OH)₁-D₃; (●), 1,25,28-(OH)₁-D₃.
Fig 4. Dose-response of (A) 1,25(OH)\textsubscript{2}16ene-23yne-D\textsubscript{3} (analogue no. 2), (B) 1,25R,26(OH)\textsubscript{3}22ene-D\textsubscript{3} (analogue no. 5), and (C) 1,25(OH)\textsubscript{2}D\textsubscript{3} (no. 1) on clonal growth of leukemic cells from patients. Bone marrow or blood mononuclear blast cells were cultured in soft agar; colonies were counted on day 10 and each point represents the mean colonies of triplicate cultures. Results are expressed as a percent of control cells not exposed to vitamin D compound (see Table 2 for ED\(_{50}\) data and diagnosis of patients). Initials of patients are on the figure.

Table 2. Effect of Vitamin D Analogs on Clonal Proliferation of Leukemic Cells From Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Colonies Formed in Absence of Vitamin D Compound</th>
<th>Inhibition of Clonal Growth (ED(_{50}) \times 10\textsuperscript{-4} mol/L) Analogs</th>
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<tbody>
<tr>
<td>C.CL</td>
<td>AML</td>
<td>145</td>
<td>NR, NR, NR</td>
</tr>
<tr>
<td>M.F</td>
<td>AML</td>
<td>54</td>
<td>NR, NR, NR</td>
</tr>
<tr>
<td>C.J</td>
<td>AML</td>
<td>182</td>
<td>1.0, 4.5, 3.8</td>
</tr>
<tr>
<td>J.A</td>
<td>AML</td>
<td>215</td>
<td>0.78, 2.7, 3.3</td>
</tr>
<tr>
<td>L.E</td>
<td>AML</td>
<td>228</td>
<td>2.3, 4.0, 3.2</td>
</tr>
<tr>
<td>C.H</td>
<td>AML</td>
<td>232</td>
<td>1.0, 2.6, 3.2</td>
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<tr>
<td>J.K</td>
<td>CML (blast phase)</td>
<td>30</td>
<td>NT, NR, NR</td>
</tr>
<tr>
<td>K.SH</td>
<td>CML (blast phase)</td>
<td>81</td>
<td>NR, NR, NR</td>
</tr>
<tr>
<td>O.L</td>
<td>CML (blast phase)</td>
<td>126</td>
<td>0.68, 2.6, 3.0</td>
</tr>
<tr>
<td>L.J</td>
<td>CML (blast phase)</td>
<td>64</td>
<td>NT, 4.5, 5.2</td>
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<tr>
<td>G.G</td>
<td>CML (blast phase)</td>
<td>219</td>
<td>0.74, 2.1, 1.9</td>
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<td>W.D</td>
<td>CML (chronic phase)</td>
<td>31</td>
<td>NT, 1.0, 4.6</td>
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<tr>
<td>K.JH</td>
<td>CML (chronic phase)</td>
<td>40</td>
<td>NT, 1.6, 1.6</td>
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<tr>
<td>H.J</td>
<td>CML (chronic phase)</td>
<td>217</td>
<td>1.9, 8, 4.6</td>
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<tr>
<td>F.RL</td>
<td>CML (chronic phase)</td>
<td>183</td>
<td>NR, NR, NR</td>
</tr>
<tr>
<td>C.CR</td>
<td>CML (chronic phase)</td>
<td>225</td>
<td>0.42, 2.7, 5.4</td>
</tr>
</tbody>
</table>

Abbreviations: CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; NT, analogue no. 2 was not tested.
determine the amount of "Ca"" absorbed (which is a measure of
ICA). BCM activity was estimated from the increase of total serum
calcium concentration, as determined by atomic absorption spectro-
photometry.

1,25(OH)₂D₃ receptor competition assays. The assay of compet-
itive binding was performed using the hydroxylapatite batch assay.²⁴²⁶ Increasing amounts of unlabeled 1,25(OH)₂D₃ or analogs
were added to a constant amount of [³H]-1,25(OH)₂D₃ and incubated
with chick intestinal cytosol or HL-60 cells. The relative
competitive index (RCI) for 1,25(OH)₂-16ene-23yne-D₃ was cal-
culated by plotting the percent maximum [³H]-1,25(OH)₂-[³H]D₃ bound
to the ordinate v [competitor]/[1,25(OH)₂-[³H]D₃] on the abscissa. The slope of the line obtained for 1,25(OH)₂-16ene-23yne-D₃ is divided by the slope of the line obtained for
1,25(OH)₂D₃; multiplication of this value by 100 results in the
RCI.²⁷ By definition, the RCI for 1,25(OH)₂D₃ is 100. For the
preparation of intestinal cytosol from vitamin D-deficient chicks, the
duodenal loop was removed after decapitation, stripped of contents,
and washed at 4°C at 0.9% NaCl solution. All subsequent steps were
carried out at 4°C. The mucosa was scraped from the serosa with
glass microscope slides and a 10% homogenate in KTED buffer (0.3
mol/L KCl, 10 mmol/L Tris, 1 mmol/L EDTA, 0.5 mmol/L
dithiothreitol, pH 7.5) was prepared.³⁸ After a low-speed centrifuga-
tion (500 g, ten minutes), cytosol from the chick intestines was
obtained by centrifugation of the supernatant at 105,000 g for one
hour. The HL-60 cells were washed two times in 1 × PBS and
resuspended in PBS.

RESULTS

Effects of vitamin D analogs on proliferation. Dose-
response effects of the various vitamin D analogs on the
clonogenic proliferation of HL-60 promyelocytes is shown in
Fig 2A. Concentrations that inhibited 50% growth (ED₅₀) are shown in tabular form (Table 1). The most potent analog
was 1α,25(OH)₂-16ene-23yne-D₃ (analogue no. 2) with an
ED₅₀ of 4 × 10⁻⁹ mol/L. The least potent analog was
1α,25,28(OH)₃-D₃ (analogue no. 9) which did not achieve an
ED₅₀. The 1α,25(OH)₂D₃ had an ED₅₀ of 16 × 10⁻⁹ mol/L.
The EM-2 myeloblasts were slightly less sensitive than
HL-60 cells to inhibition of clonal growth when grown in the
presence of the various analogs (Fig 2B, Table 1). The
1,25(OH)₂-16ene-23yne-D₃ (no. 2) was most potent (ED₅₀,
6 × 10⁻⁹ mol/L); 1α,25,28(OH)₃D₃ (no. 9) was least potent
(ED₅₀ not reached); ED₅₀ of 1,25(OH)₂D₃ was 29 × 10⁻⁹
mol/L. The monocytic cell line U937 was less sensitive to
most of the analogs as compared with HL-60 (Fig 2C, Table 1); analog 1,25(OH)₂-16ene-23yne-D₃ (no. 2), however, con-
tinued to be as potent in the inhibition of clonal growth of
U937 as it was with EM-2 and HL-60 cells. The relative rank
order of potency of the analogs on inhibition of clonal growth of
U937 remained nearly the same as they were with HL-60
and EM-2 cells. The clonal growth of KG-1 (very early
myeloblast) and BV-173 (lymphoblasts) was not inhibited
when grown in the presence of each of the 1,25(OH)₂D₃
analogs (Figs 3A and B, respectively).

Normal human myeloid clonogenic cells (GM-CFC) were
markedly stimulated in their clonal growth by the analogs
(Fig 3C). At 10⁻⁷ mol/L, enhancement of clonal growth was
about threefold for both 1,25(OH)₂-16ene-23yne-D₃ (no. 2)
and 1α,25(OH)₂D₃, twofold for analog 1α,25,28(OH)₃D₃ (no.
9), and fourfold for analog 1α,25R,26(OH)₃-22ene-D₃ (no. 5),
which was the most potent stimulator of growth. In the
absence of GM-CSF, none of the analogs was able to sustain
clonal growth of GM-CFC (data not shown).

The effect of the analogs on the clonal growth of myeloid
leukemic cells harvested from patients was examined (Fig 4,
Table 2). The subtype of leukemia of the patients are listed in
Table 2. We limited our analysis to 1,25(OH)₂-16ene-23yne-
D₃ (no. 2; most potent in inhibition of proliferation and
induction of differentiation of cells from AML lines); analog
1α,25R,26(OH)₃-22ene-D₃ (no. 5; intermediate potency); and
1,25(OH)₂D₃. Each of the compounds markedly inhibited
the clonal growth of the samples from 75% of the patients.
The 1,25(OH)₂-16ene-23yne-D₃ (no. 2) was the most potent
of the three compounds and was about two to fourfold more
active than 1,25(OH)₂D₃. The range of activity of each
1,25(OH)₂D₃ analog was similar; those samples that were
resistant to clonal inhibition by one compound were resistant
to all three. Resistant samples were from two patients with
CML in myeloid blast crisis and two patients with AML.
The range of ED$_{50}$ of the samples that were sensitive to analog no. 2 ranged between 0.42 and 2.3 x 10$^{-4}$ mol/L. We have not demonstrated that all colonies from the leukemic patients were derived from the leukemic clone. This is a shortcoming of this assay system.

**Time course of growth inhibition.** The HL-60 cells were exposed in liquid culture to 10$^{-7}$ mol/L of either 1,25(OH)$_2$-16ene-23yne-D$_3$ (no. 2) or 1,25(OH)$_2$D$_3$ for various lengths of time (0.25 to 72.0 hours), washed three times, counted, plated (2 x 10$^3$ per dish) in soft agar in the absence of a 1,25(OH)$_2$D$_3$ analog, and the numbers of colonies enumerated on day 10 of culture (Fig 5). Viability, determined by Trypan Blue exclusion, was >95% at all time points. The results are compared with cultures containing HL-60 cells treated similarly, but not exposed to analog. Fifty percent of colonies were inhibited after the cells were exposed for ten and 30 hours to 1,25(OH)$_2$-16ene-23yne-D$_3$ (no. 2) and 1,25(OH)$_2$D$_3$, respectively. By 72 hours of exposure to either compound, almost all clonal growth was inhibited.

**Effect of vitamin D analogs on differentiation of HL-60 cells.** The HL-60 cells differentiate toward macrophages when cultured in the presence of 1,25(OH)$_2$D$_3$.

Two markers of this cellular differentiation are the development of nonspecific esterase (NSE) activity and the ability to reduce nitroblue tetrazolium (NBT). We examined the ability of the 1,25(OH)$_2$D$_3$ analogs to induce differentiation of the HL-60 cells (Figs 6A and B; Table 1). The most potent inducer of differentiation was 1,25(OH)$_2$-16ene-23yne-D$_3$ (no. 2). ED$_{50}$ ~ 14 x 10$^{-9}$ mol/L; least potent was 1,25(OH)$_2$D$_3$ (no. 9); and 1,25(OH)$_2$D$_3$ had ED$_{50}$ ~ 30 x 10$^{-9}$ mol/L. Dose-response curves of each of the analogs were parallel. The ED$_{50}$ values for induction of differentiation were two to fivefold higher than the ED$_{50}$ values for inhibition of proliferation. This is observed routinely and probably reflects sensitivity in scoring the assays.

None of the analogs was able to induce differentiation of either EM-2 or KG-1 (data not shown).

**Effect of vitamin D analogs on c-myc mRNA accumulation in HL-60.** Prior studies have shown that exposure of HL-60 cells to 1,25(OH)$_2$D$_3$ decreased accumulation of c-myc mRNA. c-myc levels are a measure of the proliferation potential of these cells. Time-response studies showed that exposure of the cells for one day to either 1,25(OH)$_2$-16ene-23yne-D$_3$ (no. 2) or 1,25(OH)$_2$D$_3$ (10$^{-7}$ mol/L) decreased the concentration of c-myc mRNA by >95% (Fig 7A). Dose-response studies showed that exposure of the cells to 2 x 10$^{-8}$ mol/L 1,25(OH)$_2$-16ene-23yne-D$_3$ (no. 2) for one day decreased accumulation of c-myc mRNA by >95%; exposure to 10$^{-8}$ mol/L 1,25(OH)$_2$D$_3$ for one day decreased accumulation of c-myc mRNA to a similar degree (Fig 7B).

**Effect of vitamin D analogs on intestinal calcium absorption and bone calcium mobilization.** The major toxicity of 1,25(OH)$_2$D$_3$ is hypercalcemia, which develops because of increased intestinal calcium absorption and mobilization of bone calcium. The simultaneous measurement of the ability of the 1,25(OH)$_2$D$_3$ analogs to mediate ICA and BCM was performed by administering the analogs to chickens that were vitamin D deficient and that had been fed a zero Ca$^{2+}$ diet for 48 hours before assay. Each of these assays included a negative control designated - D; a positive control (3.25 nmol vitamin D$_3$ administered 48 hours before assay); and a series of graded, increasing doses of either 1,25(OH)$_2$D$_3$ or the test analog, which were administered 12 hours before measurement of calcium (Fig 8). At the appropriate time interval 4.0 mg $^{40}$Ca$^{2+}$ and 10 $\mu$Ci $^{45}$Ca was...
Fig 7. Modulation of c-myc mRNA expression in HL-60 by either 1,25(OH)₂D₃ (analog no. 1) or 1,25(OH)₂-16ene-23yne-D₃ (analog no. 2). (A, B) Time-response studies: HL-60 cells were cultured for various durations (0, 12, 24, 48 hours) with either 1,25(OH)₂D₃ (a) or 1,25(OH)₂-16ene-23yne-D₃ (b). (C) Dose-response studies: HL-60 cells were cultured for 24 hours with different concentrations of either 1,25(OH)₂-16ene-23yne-D₃ (analog no. 2) or 1,25(OH)₂D₃ (D₃). Lane 1, untreated cells; lane 2, 2 × 10⁻⁸ mol/L analog no. 2; lane 3, 2 × 10⁻⁹ mol/L 1,25(OH)₂D₃; lane 4, 10⁻⁸ mol/L analog no. 2; lane 5, 10⁻⁷ mol/L 1,25(OH)₂D₃; lane 6, 10⁻⁷ mol/L analog no. 2; lane 7, 10⁻⁷ mol/L 1,25(OH)₂D₃. RNA was extracted as described in the Methods section. Each lane contained 15 μg cytoplasmic RNA. When probed with c-myc cDNA (PstI-PstI fragment of exon 2), a single band was detected at 2.5 kb consistent with the known length of c-myc mRNA. A blot of Figs A and B was reprobed with β-actin to ensure equivalent amounts of RNA were present in each lane.

placed in the duodenum of the chick; serum was collected after 30 minutes and measured for ⁴⁵Ca, which is a measure of ICA and ⁶⁰Ca, a measure of BCM. In order to compare the ICA and BCM activity of the analog to that of 1,25(OH)₂D₃, a plot of log dose vs either ICA or BCM was constructed. The dose of analog required to achieve a response to either 100 pmol (ICA, Table 3) or 300 pmol (BCM; Table 4) 1,25(OH)₂D₃ was determined.

The 1,25(OH)₂D₃ was most potent in ICA; all the new vitamin D analogs had activity ranging from 10% to only 0.6% that of 1,25(OH)₂D₃ in ICA (Table 3, Fig 9). The 1,25(OH)₂-16ene-23yne-D₃ (no. 2) which is the most potent inducer of differentiation and inhibitor of proliferation of HL-60 cells, was only 3% as active as 1,25(OH)₂D₃ in the ICA assay.

For the BCM assay, we confined our analysis to the five vitamin D compounds that were most potent in induction of differentiation and inhibition of proliferation of HL-60 cells (Fig 8, Table 4). The 1,25(OH)₂D₃ was most potent in BCM; all the vitamin D analogs were only 2% to 10% as potent as 1,25(OH)₂D₃ in BCM; 1,25(OH)₂-16ene-23yne-D₃ (no. 2) was 50-fold less potent than 1,25(OH)₂D₃.

Ability of 1,25(OH)₂D₂-16ene-23yne-D₃ to compete with 1,25(OH)₂D₃ for binding to 1,25(OH)₂D₃ receptors. The ability of 1,25(OH)₂-16ene-23yne-D₃ (no. 2) (most potent inhibitor of proliferation and inducer of differentiation of leukemic cells) to compete with 1,25(OH)₂[³H]D₃ for binding to the 1,25(OH)₂D₃ receptors from both chick intestine and HL-60 cells are shown in Fig 9. Competition was quantitated by measuring the decrease in specific binding of a fixed concentration of 1,25(OH)₂[³H]D₃ as increasing concentrations of analog were added to an in vitro incubation. The RCI obtained for 1,25(OH)₂-16ene-23yne-D₃ (no. 2) was a mean 98 ± 17 (SD) when using chick intestinal derived receptors and a mean 60 ± 7.6 (SD) when using HL-60 derived receptors (data represent mean of three and four experiments for chick intestinal cells and HL-60 cells, respectively).

DISCUSSION

This study examined eight new 1,25(OH)₂D₃ analogs (see Fig 1); seven of the eight analogs were the same or more potent than 1,25(OH)₂D₃ in their abilities either to induce differentiation or to inhibit proliferation of leukemic cells
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In contrast, these compounds were 10 to 50-fold less potent in their ability to stimulate intestinal absorption of calcium or mobilization of calcium from bone of vitamin D-deficient chicks (Tables 3 and 4). The analog 1,25(OH)₂-D₃ (no. 1) was the most potent modulator of growth and differentiation of leukemic cells in most of the assays; it was four to 14-fold more active than 1,25(OH)₂D₃. In contrast, this analog was 30 and 50-fold less potent in ICA and BCM, respectively as compared with 1,25(OH)₂D₃. Although the potency of the other analogs varied, their range of activities were similar. In contrast to their effect on leukemic cells, each of the analogs stimulated the clonal growth of normal human myeloid stem cells (GM-CFC); the stimulation was in the range of two to fourfold and occurred in the presence of maximally stimulating concentrations of recombinant GM-CSF.

Because of the marked potency of 1,25(OH)₂-16ene-23yne-D₃ (no. 2), several additional experiments focused on this compound. Exposure to 1,25(OH)₂-16ene-23yne-D₃ (no. 2) (10⁻⁷ mol/L) for ten hours was sufficient to inhibit by 50% the clonal proliferation of HL-60 cells. A similar duration of exposure of 1,25(OH)₂-16ene-23yne-D₃ (no. 2) decreased by about 80% the accumulation of c-myc mRNA in HL-60 cells. 1,25(OH)₂-16ene-23yne-D₃ (no. 2) was about tenfold more potent than 1,25(OH)₂D₃ in decreasing levels of c-myc. Expression of the c-myc is associated with the potential of cellular proliferation.³⁷ We⁷ and others⁶ have previously shown that the HL-60 have about 4,000 receptors for 1,25(OH)₂D₃ per cell with a dissociation constant for the ligand in the range of 5 × 10⁻⁹ mol/L. Further studies showed that the ability of the 1,25(OH)₂D₃ analogs to bind to 1,25(OH)₂D₃ receptors in general paralleled their ability to induce differentiation of HL-60 cells, which is consistent with these compounds mediating their effects through the 1,25(OH)₂D₃ receptors. We performed experiments to determine the relative effectiveness of binding to the chick intestinal mucosa receptor and HL-60 receptor for 1,25(OH)₂D₃. The results were expressed as RCI, where the RCI is defined as 100 for 1,25(OH)₂D₃.

![Fig 8. Effect of 1,25(OH)₂-16ene-23yne-D₃ (no. 2) or 1,25(OH)₂D₃ (no. 1) on intestinal calcium absorption (ICA) and bone calcium mobilization (BCM). The 1,25(OH)₂-16ene-23yne-D₃ (no. 2) and 1,25(OH)₂D₃ were given intramuscularly to vitamin D deficient chicks 12 hours before assay. Results are expressed as mean ± SE in groups of seven chicks. Each assay included a negative control (−D) and a positive control (3.25 mol vitamin D₃).](from_east)
NOVEL VITAMIN D ANALOGS ON LEUKEMIC CELLS

Table 4. Effect of Vitamin D Analogs on Bone Calcium Mobilization

<table>
<thead>
<tr>
<th>No.</th>
<th>Analog Name</th>
<th>ED-50 of HL-60 (x 10^-8 mol/L)</th>
<th>Bone Calcium Mobilization†</th>
<th>Dose of Analog to Achieve Response Equivalent to 300 pmol 1,25(OH)2D3</th>
<th>Ratio [1,25(OH)2D3]</th>
<th>[Analog Dose]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,25-(OH)2-D3</td>
<td>16</td>
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<td></td>
<td>300</td>
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<tr>
<td>2</td>
<td>1,25-(OH)2-16ene-23yne-D3</td>
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<td>0.02</td>
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<td>1,25-(OH)2-23yne-D3</td>
<td>7</td>
<td>5,300</td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1,25S,26-(OH)3-D2</td>
<td>7</td>
<td>5,000</td>
<td></td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1,25R,26-(OH)3-22ene-D3</td>
<td>8</td>
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<td></td>
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</tr>
<tr>
<td>6</td>
<td>1,25S,26-(OH)3-22ene-D3</td>
<td>8</td>
<td>3,000</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

†The results presented in the table for BCM are derived from dose response studies for each analog similar to that presented in Fig 9.

The vitamin D analog was administered to vitamin D deficient chicks 12 hours before the assay. The chicks were anesthetized and 4.0 mg of 45Ca and 10 Ci 45Ca were placed in the duodenum. After 30 minutes, sera were sampled for 45Ca.

ED-50 represents dose of vitamin D analog that inhibited 50% of HL-60 cells in the clonogenic assay; see Table 1.

Cells was 60 ± 7.6, which is significantly different from that of the RCI of 1,25(OH)2D3, which is 100. These results infer that the specificity of the ligand binding domains for the 1,25(OH)2D3 receptor present in the HL-60 is different from that present in the chick intestine. No information is yet available comparing the biochemical properties of 1,25(OH)2D3 receptors present in different species or in differing target tissues in the same species.

The seco-steroid 1,25(OH)2D3 is the physiological mediator of calcium metabolism (Fig 1). We found that specific modification of the side chain and the D ring can enhance the potency of the analog. Addition of a triple bond between C-23, C-24, and addition of a double bond between C-16, C-17 [as in 1,25(OH)2-16ene-23yne-D3] enhanced differentiation of HL-60 about fourfold over 1,25(OH)2D3. Elimination of the D ring double bond and having only the triple bond between C-23, C-24 [as in 1,25(OH)2-23yne-D3] (no. 3) resulted in a twofold greater potency compared with 1,25(OH)2D3. A double bond at C-22, C-23 and the addition of a hydroxyl group to C-26 also increased the potency [as in 1,25R,26-(OH)3-22ene-D3 (no. 4) or 1,25S,26-(OH)3-22ene-D3 (no. 5)] twofold as compared with 1,25(OH)2D3.

The new analogs had an ICA activity ranging from 0.6% to 10% of that of 1,25(OH)2D3. The presence of a triple bond at C-23, C-24 [as in 1,25(OH)2-23yne-D3 (no. 3)] or a double bond at C-22, C-23 and an additional hydroxyl moiety at C-26 (no. 4, 5, 6) decreased the ICA by 30 to 150-fold as compared with 1,25(OH)2D3. Addition of a hydroxyl group to C-23 or C-28 [as in 1,23S,25(OH)3-D3 (no. 7) or 1,25,28(OH)3-D3 (no. 9)] decreased the ICA only eight to tenfold compared with 1,25(OH)2D3.

Recently, several other vitamin D compounds have been described that are either of greater or equivalent potency as 1,25(OH)2D3 in the induction of differentiation or the inhibition of proliferation of HL-60 cells. We and others have found that the introduction of fluoro groups either at position C-24 or C-26 or C-27 to 1,25(OH)2D3 enhances by four to sevenfold the ability of these analogs to induce differentiation of HL-60 cells. Extension of the vitamin D3 side-chain by one carbon [as in 24-homo-1,25(OH)2D3 and 26-homo 1,25(OH)2D3] resulted in a tenfold increase in the differentiation inducing potency as compared with 1,25(OH)2D3.11

The 26-homo analog is more active and the 24-homo analog is less active than 1,25(OH)2D3 in BCM.

Several factors are known to influence the biological activity of analogs of 1,25(OH)2D3 including: (a) their availability to cells in the presence of vitamin D-binding proteins present in the serum; (b) their ability to diffuse into cells; (c) their binding affinity for the 1,25(OH)2D3 receptors; and (d) their metabolism and degradation. We do not know which of these variables may be operative in defining differences in biological effectiveness of our new analogs. Differences in availability to the cell is unlikely because all experiments were presumably performed in the presence of 1,25(OH)2D3.
excess vitamin D binding proteins; diffusion of 1,25(OH)2D3 and vitamin D analogs is usually not thought to be rate limiting. Perhaps our new analogs are not as efficiently metabolized and degraded in hematopoietic cells as 1,25(OH)2D3,33,34 which may account for their marked hematopoietic potency even when they have a low RCI.

Our current studies show that >10-9 mol/L 1,25(OH)2D3 is required for inhibition of proliferation and induction of differentiation of HL-60 cells. Sera concentrations of 1,25(OH)2D3 of 10-9 mol/L in humans will produce hypercalcemia thereby limiting potential therapeutic use of the seco-steroid. Our novel compounds are more potent than 1,25(OH)2D3 in their induction of differentiation and inhibition of clonal proliferation of leukemic cells without inhibiting normal myeloid clonal growth; also they possessed much lower BCM and ICA values than 1,25(OH)2D3 suggesting less potential for causing hypercalcemia. Preliminary in vivo murine studies show that 1,25(OH)2D3 produced at least 20-fold less hypercalcemia as compared with 1,25(OH)2D3 (data not shown).

ACKNOWLEDGMENT

We would like to thank Suzanne Bookstaver, Carol Leitz, and Regina Simon for their secretarial help and June Bishop, Tsuneko Akashi, and Grace Jones for excellent technical assistance.

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Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone mobilization

JY Zhou, AW Norman, M Lubbert, ED Collins, MR Uskokovic and HP Koeffler