19-nor Vitamin-D Analogs: A New Class of Potent Inhibitors of Proliferation and Inducers of Differentiation of Human Myeloid Leukemia Cell Lines

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We have studied the in vitro biological activities and mechanisms of action of 1,25-dihydroxyvitamin D3 (1,25D3) and nine potent 1,25D3 analogs on proliferation and differentiation of myeloid leukemia cell lines (HL-60, retinoic acid-resistant HL-60 [RA-res HL-60], NB4 and Kasumi-1). The common novel structural motif for almost all the analogs included removal of C-19 (19-nor); each also had unsaturation of the side chain. All the compounds were potent; for example, the concentration of analogs producing a 50% clonal inhibition (ED50) ranged between 1 x 10^-9 to 4 x 10^-11 mol/L when using the HL-60 cell line. The most active compound [1,25(OH)2-16,23-diene-26-trifluoro-19-nor-cholecalciferol (Ro 25-9716)] had an ED50 of 4 x 10^-11 mol/L; in contrast, the 1,25D3 produced an ED50 of 10^-9 mol/L with the HL-60 target cells. Ro 25-9716 (10^-7 mol/L, 3 days) was a strong inducer of myeloid differentiation because it caused 92% of the HL-60 cells to express CD11b and 75% of these cells to reduce nitroblue tetrazolium (NBT). This compound (10^-6 mol/L, 4 days) also caused HL-60 cells to arrest in the G1 phase of the cell cycle (88% cells in G1 v 48% of the untreated control cells). The p27kip-1, a cyclin-dependent kinase inhibitor which is important in blocking the cell cycle, was induced more quickly and potently by Ro 25-9716 (10^-7 mol/L, 0 to 5 days) than by 1,25D3, suggesting a possible mechanism by which these analogs inhibit proliferation of leukemic growth. The NB4 promyelocytic leukemia cells cultured with the Ro 25-9716 were also inhibited in their clonal proliferation (ED50, 5 x 10^-11 mol/L) and their expression of CD11b was enhanced (80% positive [10^-9 mol/L, 4 days] v 27% untreated NB4 cells). Moreover, the combination of Ro 25-9716 (10^-9 mol/L) and all-trans retinoic acid (ATRA, 10^-7 mol/L) induced 92% of the NB4 cells to reduce NBT, whereas only 26% of the cells became NBT positive after a similar exposure to the combination of 1,25D3 and ATRA. Surprisingly, Ro 25-9716 also inhibited the clonal growth of poorly differentiated leukemia cell lines (RA-res HL-60 [ED50, 4 x 10^-9 mol/L] and Kasumi-1 [ED50, 5 x 10^-10 mol/L]). For HL-60 cells, Ro 25-9716 markedly decreased the percent of the cells in S phase of the cell cycle and increased the expression of the cyclin-dependent kinase inhibitor, p27kip-1. In summary, 19-nor vitamin D3 compounds strongly induced differentiation and inhibited clonal proliferation of various myeloid leukemia cell lines, suggesting a therapeutic niche for their use in myeloid leukemia.

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MATERIALS AND METHODS

Cell lines. HL-60, retinoic acid-resistant HL-60 (RA-res HL-60), NB4, and Kasumi-1 cell lines were grown in RPMI 1640 medium (GIBCO Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (GIBCO) under standard culture conditions. The NB4 promyelocytic leukemia cell line was provided by Dr Lanotte (INSERM, Hospital Saint-Louis, Paris, France).19 RA-res HL-60, which is a subline of HL-60 cells, is resistant to both all-trans retinoic acid (ATRA) and 1,25D3 and has a mutation at codon 411 of the retinoic acid receptor alpha gene (kindly provided by Dr Gallagher, Albert Einstein Cancer Center, NY).20 Kasumi-1 cell line is an undifferentiated...
myeloid leukemia cell line that carries a t(8;21)(q22;q22) chromosomal abnormality. 1,25D$_3$ analogs. The parental compound [1α,25(OH)$_2$D$_3$] and its analogs were dissolved in absolute ethanol at $10^{-3}$ mol/L and stored at −20°C. The concentration of the analogs was determined by measurements of ultraviolet absorbance using their molar extinction coefficient at 264 nmol/L. Dilutions were made in the same tissue culture medium used for growing the leukemia cell lines. All manipulation with vitamin D analogs were performed in subdued light. The maximum concentration of ethanol used in this study had no influence on cell growth (data not shown). The simplified code names and structures of the 1,25D$_3$ analogs are shown in Fig 1.

Cytotoxicity test. HL-60 (10$^5$/mL) cells were incubated in liquid culture for 3 days with various concentrations (10$^{-8}$ to 10$^{-9}$ mol/L) of 1,25D$_3$ or Ro 25-9716. After the culture, cell viability was evaluated by staining with trypan blue. Inhibition of exponentially growing cells was estimated by counting the cell number after HL-60, RA-res HL-60, NB4, or Kasumi-1 cells (seeded at 5 × 10$^4$/mL) were treated with 10$^{-7}$ mol/L of either 1,25D$_3$ or Ro 25-9716 for 4 days.

Colony formation in soft agar. Cells were seeded in a two-layer soft agar system as previously described. 22 The lower layer consisted of 0.5% agar in which the test substances were mixed; upper layer was 0.3% agar (Difco Laboratories, Detroit, MI) in which 1,000 to 5,000 cells were mixed per plate. All experimental points were performed in triplicate. All plating experiments were repeated at least twice. After 10 days of incubation at 37°C in a humidified atmosphere containing 5% CO$_2$ in air, colonies (>40 cells) were counted using an inverted microscope.

Studies of induction of differentiation. Differentiation of HL-60 and NB4 cells was assessed by their abilities to produce superoxide as measured by reduction of nitroblue tetrazolium (NBT), 23 by morphology as detected on cytopsin preparations stained with Diff-Quick Stain Set (Baxter Healthcare Corp, Miami, FL), and by analysis of expression of CD11b surface marker by flow cytometry.

Pulse-exposure experiments. HL-60 cells were incubated in liquid culture for various durations with 10$^{-9}$ mol/L of either 1,25D$_3$ or Ro 25-9716. After incubation, cells were carefully washed twice, once with phosphate-buffered saline and once with media before being counted and plated into 24-well plates for soft agar colony assay. Some HL-60 cells were plated in the liquid culture without compounds after two times wash-out; terminal differentiation of HL-60 cells was evaluated by NBT test at the third day of the culture.

Cell cycle analysis. The cell cycle was analyzed by flow cytometry after 96 hours of incubation of cells (5 × 10$^4$/mL) either with or without analogs (10$^{-8}$ mol/L) as described. 24 Briefly, the cells were fixed in cold ethanol and incubated for 30 minutes at 4°C in the dark with a

Fig 1. Structure and code name of the novel vitamin D3 analogs examined in this study.
solution of 50 mg/mL propidium iodide, 1 mg/mL RNase (Sigma, Saint-Louis, MO), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CELLFIT program (Becton Dickinson, Mountain View, CA), whereby the S phase was calculated with an RFit model.

Expression of p27kip-1. Western blot analysis was performed with polyclonal rabbit anti-p27 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal antiamyeloperoxidase (MPO) antibody, which recognizes the 55-kD MPO protein. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as previously described. Briefly, proteins (40 µg) were size fractioned under denaturing conditions on 12.5% SDS-running gel and transferred to Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). The protein was detected using the enhanced chemiluminescence system from Amersham (Arlington Heights, IL).

RESULTS
Effects of 1,25D₃ analogs on clonal growth of leukemia cell lines. All analogs of 1,25D₃ were tested in a dose-response fashion (10⁻¹¹ to 10⁻⁶ mol/L) and each was active in the clonal inhibition of HL-60 growth in soft agar. Dose-response curves were prepared (data not shown), and the concentration at which 50% of the colonies were inhibited (ED₅₀) by each analog was calculated (Table 1). For all analogs, the ED₅₀ ranged from 6 × 10⁻¹⁰ to 4 × 10⁻¹¹ mol/L; by comparison, the parental 1,25D₃ achieved an ED₅₀ of 1 × 10⁻⁹ mol/L. The most potent analog (Ro 25-9716, 1,25S-dihydroxy-16,23E-diene-26-trifluoro-19-nor-cholecalciferol) had an ED₅₀ of 4 × 10⁻¹¹ mol/L, which was 25-fold more potent than 1,25D₃ (Fig 2). Likewise, this analog (Ro 25-9716) was examined for its ability to inhibit clonal growth of the RA-res HL-60, NB4, and Kasumi-1 cell lines (Fig 2). The clonal growth of RA-res HL-60 was not inhibited by 1,25D₃, but remarkably, Ro 25-9716 did suppress the clonal growth of RA-res HL-60 cells (ED₅₀, 7 × 10⁻⁹ mol/L). The clonal growth of both NB4 and Kasumi-1 cells was inhibited by both 1,25D₃ and Ro 25-9716, but Ro 25-9716 was more active

| Table 1. Effects of Vitamin D₃ Analogs on Clonal Growth of HL-60 Cells |
|-----------------------------|---------------------------|
| Chemical Name               | Code Name                | ED₅₀ (mol/L) |
| 1,25-(OH)₂ D₃              | 1,25D₃           | 1 × 10⁻⁹   |
| 1,25S-(OH)₂-16,23-diene-26-F₆-19-nor D₃ | Ro 25-9716 | 4 × 10⁻¹¹ |
| 1,25-(OH)₂-16-ene-23-yne-26-F₆-19-nor D₃ | Ro 26-3016 | 6 × 10⁻¹¹ |
| 1,25-(OH)₂-16-ene-19-nor D₃   | Ro 25-7290 | 3 × 10⁻¹⁰ |
| 1,25-(OH)₂-16,23Z-diene-19-nor D₃ | Ro 25-7463 | 4 × 10⁻¹⁰ |
| 1,25-(OH)₂-16-ene-23-yne-19-nor D₃   | Ro 25-7856 | 5 × 10⁻¹⁰ |
| 1,25-(OH)₂-16,23Z-diene-26,27-F₆-19-nor-20-epi D₃ | Ro 26-3884 | 5 × 10⁻¹⁰ |
| 1,25-(OH)₂-16,23E-diene-19-nor D₃   | Ro 25-7555 | 6 × 10⁻¹⁰ |
| 1,25-(OH)₂-16,23E-diene-26,27-F₆ D₃ | Ro 25-5818 | 6 × 10⁻¹⁰ |

Each compound was tested in log intervals in dose-response studies (10⁻¹¹ to 10⁻⁷ mol/L); means ± SD were determined and plotted on semilog paper. The dose that produced 50% inhibition of colony formation (ED₅₀) was calculated. Each experimental point had three plates per experiment; the SD was always less than 10%.
(ED₅₀: NB4, 5 × 10¹¹ mol/L; Kasumi-1, 5 × 10⁸ mol/L) than 1,25D₃ (ED₅₀: NB4, 1 × 10⁹ mol/L; Kasumi-1, 6 × 10⁻¹⁰ mol/L).

**Cytotoxicity test.** Viability of HL-60 cells was 98% in liquid culture without 1,25D₃ analogs. Trypan blue–positive HL-60 cells increased to 28% after their exposure to Ro 25-9716 (10⁻⁸ mol/L, 3 days), as compared with 8% after culture with 1,25D₃ (10⁻⁸ mol/L, 3 days). Morphologically, the dead cells that appeared after exposure to Ro 25-9716 were apoptotic. In liquid culture, Ro 25-9716 (10⁻⁷ mol/L, 4 days) inhibited growth of the HL-60 cells, but 1,25D₃ (10⁻⁷ M, 4 days) did not (cell counts were 64% or 99% of controls [no compounds], respectively). Cell counts of RA-res HL-60 (75% v 136%), NB4 (68% v 107%), and Kasumi-1 (38% v 54%) were also decreased with Ro 25-9716 as compared with 1,25D₃ (percentage of control cultures after treatment with Ro 25-9716 v 1,25D₃, respectively). Increase of apoptotic cells was not observed in these three cell lines after exposure to Ro 25-9716.

**Pulse-exposure experiments.** The HL-60 cells were cultured with vitamin D₃ compound (10⁻⁹ mol/L) for different durations (1 to 4 days), thoroughly washed, counted (>99% of the population was viable), plated in soft-agar, and colonies were enumerated 10 days later. More than 50% of the HL-60 cells were inhibited by 1 day of exposure to analog Ro 25-9716 (10⁻⁹ mol/L), and a 3-day exposure inhibited more than 90% of the clonogenic cells, suggesting that this analog was capable of mediating an irreversible inhibition of growth of these cells (Fig 3). Notably, 1,25D₃ (10⁻⁹ mol/L) inhibited only 21% of the clonogenic cells after 3 days of exposure. Terminal differentiation was observed in the cells exposed to Ro 25-9716 (10⁻⁹ mol/L, 4 days) (10% NBT-positive cells). On the other hand, less than 1% of NBT-positive HL-60 cells were observed after the cells were exposed to 1,25D₃ (10⁻⁹ mol/L, 4 days).

**Effect of 1,25D₃ analogs on differentiation of leukemia cell lines.** A 3-day exposure of HL-60 cells to either 1,25D₃ or Ro 25-9716 (10⁻⁹ mol/L) resulted in 10% and 83% NBT-positive

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![Fig 3. Pulse-exposure of HL-60 cells to either 1,25D₃ (□) or Ro-25-9716 (●)]. HL-60 cells were exposed for various durations to the vitamin D₃ compounds (10⁻⁹ mol/L). The cells were then thoroughly washed three times, counted, plated (1,000 cells/well) into soft agar, and colonies were counted 14 days after plating. Each point represents the mean of three experiments with triplicate dishes per point.

![Fig 4. Comparison of the differentiation-inducing activities of 1,25D₃ and Ro-25-9716. (A) NBT reduction activities. HL-60 cells were cultured with various concentrations (10⁻¹¹ to 10⁻⁷ mol/L) of either 1,25D₃ or Ro 25-9716 for 3 days and differentiation was determined by NBT reduction. (□), 1,25D₃; (●), Ro 25-9716. (B) Expression of CD11b antigens on HL-60 cells. Cells were treated for 3 days with different concentrations (10⁻⁷ to 10⁻⁸ mol/L) of either 1,25D₃ or Ro 25-9716, and then analyzed by fluorescence-activated cell sorting (FACS) for expression of CD11b. (□), 1,25D₃; (●), Ro 25-9716.](https://www.bloodjournal.org/content/141/24/2444/F11)
cells, respectively (Fig 4A). With the same culture conditions, 2% and 92% of the HL-60 cells expressed CD11b protein in the presence of either 1,25D$_3$ or Ro 25-9716, respectively (Fig 4B). Also, with the same culture conditions, Ro 25-9716 at 10$^{-7}$ mol/L induced 50% of NB4 cells to express CD11b, whereas approximately 40-fold more 1,25D$_3$ was required to induce a similar degree of differentiation (Fig 5A). ATRa alone (10$^{-7}$ mol/L × 3 days) induced less than 5% of NB4 cells to differentiate as measured by NBT when Ro 25-9716 was combined with ATRA; it markedly enhanced differentiation (ED$_{50}$, 2 × 10$^{-10}$). Although the Kasumi-1 cells showed no morphological differentiation when cultured with either 1,25D$_3$ or Ro 25-9716 (10$^{-9}$ mol/L, 3 days) (data not shown), 9% and 43% of these cells became CD11b positive after their incubation with either 1,25D$_3$ or Ro 25-9716 (10$^{-9}$ mol/L, 3 days), respectively (Fig 6). The RA-res HL-60 neither differentiated morphologically nor expressed CD11b surface markers after culture with either 1,25D$_3$ or Ro 25-9716 (10$^{-7}$ mol/L, data not shown).

**Cell cycle analysis.** Effect of analog Ro 25-9716 on the cell cycle of the leukemia cell lines was examined. The HL-60 cells had a significant increased number of cells (88%) in the G$_1$ phase of the cell cycle after their exposure to Ro 25-9716 (10$^{-9}$ mol/L, 96 hours) as compared with wild-type HL-60 cells (48% in G$_1$, Fig 7). The NB4 cells also had an increased number of cell (63%) in G$_1$ after their exposure to Ro 25-9716 (10$^{-7}$ mol/L, 96 hours) as compared with wild-type NB4 cells (47%). The Kasumi-1 cells had a small percentage increase (76%) in G$_1$ and a small decrease (16%) in S phase after their exposure to Ro 25-9716 (10$^{-9}$ mol/L, 96 hours), as compared with wild-type Kasumi-1 cells (71% in G$_1$ and 21% in S, data not shown). In contrast, the RA-res HL-60 cells showed no increase in the number of cells in the G$_1$ phase after incubation with either 1,25D$_3$ or Ro 25-9716 (10$^{-6}$ mol/L, 96 hours, data not shown).

**Induction of expression of p27$^{kip-1}$.** The HL-60 cells did not express easily detectable p27$^{kip-1}$ before exposure to the vitamin D$_3$ compounds. However, these cells contained low levels of p27$^{kip-1}$ after 1-day incubation with Ro 25-9716 (10$^{-7}$ mol/L),...
and these levels markedly increased on day 2 of culture. In contrast, the expression of p27kip-1 did not increase until the third day of incubation with 1,25D$_3$ (10$^{-7}$ mol/L), and even at days 4 and 5 of culture, the intensity of expression was generally weak (Fig 8). The other three cell lines (RA-res HL-60, NB4, and Kasumi-1) weakly expressed p27 protein before exposure to the vitamin D$_3$ compounds, and none of these cell lines had an increased level of p27 protein after exposure to vitamin D$_3$ compounds (data not shown).

**DISCUSSION**

The 1,25D$_3$ and its analogs inhibited the proliferation of various cancer subtypes in vitro and in vivo.$^{7,18}$ Previously, our data showed that 1,25D$_3$ analogs that had the removal of their C-19 moiety (19-nor 1,25D$_3$ analogs) were extremely active against prostate cancer cell lines that were resistant to other vitamin D$_3$ analogs.$^{27}$ Moreover, the 19-nor analog with the code name LH [1,25-(OH)$_2$-16-ene-23-yne-26,27-F$_6$-19-nor-D$_3$] was the most potent inhibitor of clonal proliferation of breast and prostate cancer cell lines.$^{28}$ Therefore, we synthesized additional novel 19-nor 1,25D$_3$ analogs and examined their biological effects on myeloid leukemia cell lines. This study showed that all nine novel analogs of 1,25D$_3$ had a remarkable ability to inhibit the clonal growth of HL-60 cells. The most potent analog (ED$_{50}$, 4 × 10$^{-10}$ mol/L) was Ro-25-9716 (1,25S-dihydroxy-16,23E-diene-26-trifluoro-19-nor cholecalciferol) and therefore, we focused on the scope of activity of this analog compared with 1,25D$_3$ on various myeloid leukemia cell lines. The Ro 25-9716 was active against all four myeloid leukemia cell lines (HL-60, RA-res HL-60, NB4, and Kasumi-1). This compound strongly inhibited clonal proliferation and induced differentiation of HL-60 cells; its effect in some respects may be more potent than that of the 20-epi-1,25D$_3$ analog known as
Western blot analysis.

VITAMIN D3 ANALOGS AND MYELOID LEUKEMIA

25-9716 (10\(^{-9}\) mol/L) from the cells were analyzed for expression of p27kip-1 or MPO using clonal growth of HL-60 cells occurred with Ro 25-9716 (10\(^{-9}\) mol/L) alone. 

The differentiation of the promyelocytic leukemic NB4 cells.30 This cell line is an interesting model to investigate novel agents for this subtype of acute myeloid leukemia. Our results showed that vitamin D\(_3\) analogs (especially Ro 25-9716) may offer a therapeutic approach to the t(8;21) leukemias because Ro 25-9716 strongly suppressed clonal growth of the Kasumi-1 cells and induced expression of CD11b on their cell surfaces. Although we could detect neither morphological differentiation (data not shown) nor NBT reduction of these cells, further testing of these vitamin D\(_3\) compounds on samples from patients with t(8;21) leukemia may provide further support for their use.

The HL-60 cells were arrested in the G\(_1\) stage of the cell cycle after their culture with Ro 25-9716 (10\(^{-8}\) mol/L, 96 hours). The size of this G\(_1\)-arrested population induced by Ro 25-9716 (G\(_1\) phase, 88%) was much greater than that produced by exposure to 1,25D\(_3\) (G\(_1\),42%). Recently, Wang et al32 suggested that the CDKI p27\(^{kip-1}\) is a strong candidate for the cell cycle regulator that blocks the entry into S phase in 1,25D\(_3\)-treated HL-60 cells. Therefore, we speculated that an increased expression of p27\(^{kip-1}\) protein would be associated with the prominent G\(_1\) arrest that occurred after the cells were incubated with Ro 25-9716. Western blot analysis showed that the p27\(^{kip-1}\) protein was expressed more rapidly and strongly after exposure of HL-60 cells to Ro 25-9716 compared with 1,25D\(_3\); this induction of expression of p27\(^{kip-1}\) was correlated with the strong G\(_1\) arrest induced by Ro 25-9716. A recent study has shown that overexpression of p27\(^{kip-1}\) could induce apoptosis in human carcinoma cell lines, melanoma cell line, lung fibroblasts, and rat fibroblast cell line.33 This study suggested that expression of p27\(^{kip-1}\) is associated with not only regulation of cell cycle but also apoptotic death of mammalian cells. Therefore, it is reasonable that expression of p27\(^{kip-1}\) was enhanced by Ro 25-9716 only in the HL-60 cell line that showed apoptotic death of the cells after treatment with Ro 25-9716. Our results support this hypothesis that the p27\(^{kip-1}\) protein may be one of the principle mediators of the antiproliferative activity of the vitamin D\(_3\) compounds by both blocking entry of the leukemic cells into the S phase and inducing apoptosis of these cells. However, this cannot be the complete explanation, because Ro 25-9716 inhibited the growth of NB4, RA-res HL-60, and Kasumi-1 cells but it did not cause an increased expression of p27\(^{kip-1}\) in these cells.

Of particular interest, the clonal growth of the RA-res HL-60 cells was inhibited by Ro 25-9716 but not by 1,25D\(_3\). This suggests that this novel analog has not only a quantitatively enhanced antileukemic activity compared with 1,25D\(_3\), but it also has a qualitative difference in activity. Perhaps the vitamin D receptors, when bound to Ro 25-9716, interact with a novel set of genes that regulate cellular proliferation. Further studies, such as the identification of transcriptional factors regulated...
differentially in either these or similarly resistant HL-60 cells would help to elucidate the mechanism leading to an expanded scope of activities of Ro 25-9716.

In summary, we have synthesized and identified a group of 19-nor 1,25D3 analogs (especially Ro 25-9716) with potent effects on proliferation and differentiation of myeloid leukemia cell lines in vitro. These interesting analogs will be studied for their ability to control myeloid leukemias in vivo.

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