ABSTRACT

Vitamin D₃ [1,25-dihydroxyvitamin-D₃ (1,25(OH)₂D₃)] modulates the proliferation and differentiation of many cell types. Analogs of 1,25(OH)₂D₃ that have greater potency may become adjuvant therapy for breast and prostate cancers, myelodysplastic syndrome, acute myelogenous leukemia in remission and other cell types, especially in the setting of low disease burden. A new class of analogs of 1,25(OH)₂D₃ has been synthesized that has a novel 19-nor motif, as well as incorporating many structural elements previously shown to increase potency. These analogs were examined for their effects on prostate cancer cell lines (PC-3, LNCaP, and DU 145), a human breast cell line (MCF-7), and an acute myeloid leukemia cell line (HL-60). Dose-response clonogenic studies showed that each of these analogs had more potent antiproliferative activities against the cancer cells than 1,25(OH)₂D₃, and 1,25(OH)₂-16,23Z-diene-26,27-bishomo-19-nor-D₃; Ro 27-2014 was the most potent analog (10-fold increased activity compared to 1,25(OH)₂D₃). Further studies were performed using Ro 27-2014. Pulse-exposure studies showed that a 5-day pulse-exposure to Ro 27-2014 (10⁻⁷ M) in liquid culture was adequate to achieve a 50% inhibition of MCF-7 clonal growth in soft agar in the absence of the analog, suggesting that the growth inhibition mediated by the analog was irreversible. Cell cycle analyses using MCF-7 cells showed that Ro 27-2014 (10⁻⁷ M for 4 days) induced a significant increase in the number of cells in Gő-G₁ (72.8 ± 8.9% versus 49.9 ± 3.5% in control cells), with a concomitant decrease in the percent of cells in S phase (13.1 ± 6.2% versus 35.8 ± 3.5% in control cells). The chief toxicity of vitamin D₃ compounds is hypercalcemia, and therefore, we examined calcemic activity of Ro 27-2014 in mice and found it not to induce hypercalcemia at doses of 0.05 µg i.p. three times per week. In contrast, the same dose of a 19-nor vitamin D₃ compound with 6 fluorones on the side chain (1,25(OH)₂-16-ene-23-yne-26,27-F₆-19-nor-D₃), although also having potent anticancer activity, caused severe hypercalcemia (18 mg/dl). In summary, 19-nor vitamin D₃ compounds with desaturation and lengthening of their side chains result in a series of compounds with a good therapeutic index, having potent antiproliferative activity and low toxicity.

INTRODUCTION

Breast and prostate cancers are two of the most common malignant diseases in the United States. Current chemotherapy of cancer is mostly based on agents that are toxic to the cells. Induction of differentiation represents an alternative to conventional therapies. For example, the treatment of acute promyelocytic leukemia with all-trans retinoic acid demonstrated differentiation of malignant promyelocytes and has become one of the standard therapies (1, 2).

1,25(OH)₂D₃, which belongs to the seco-steroid hormone family, is a key regulator of calcium homeostasis (3). 1,25(OH)₂D₃ initiates genomic responses through a specific nuclear vitamin D₃ receptor, which interacts with a vitamin D₃-responsive element and regulates specific gene transcription (4). It inhibits the growth and induces differentiation of many types of cancer cells in vitro and in vivo, including those from the prostate (5–8), breast (9–13), blood (14–17), colon (18, 19), squamous skin (20), and brain (21). However, the calcemic side effects of 1,25(OH)₂D₃ prevent its application as a therapeutic agent (22). Synthesis of analogs of 1,25(OH)₂D₃ with potent antiproliferative and differentiation activities against malignant cells decreased risk of inducing hypercalcemia. Several analogs of 1,25(OH)₂D₃ have prominent antiproliferative effects against cancer cells and cells without resulting in lethal hypercalcemia when administered in vivo at pharmacologically active doses.

Previously, we have studied the in vitro biological activities of 19-nor-hexafluoride analogs of vitamin D₃ on the proliferation and differentiation of cell lines from breast and prostate cancers and leukemias (27–29). In these studies, these compounds, which have six fluorones on their side chain, demonstrated strong inhibition of growth and induction of differentiation of cancer cells; especially potent was compound LH (also known as Ro 25-6760). However, compound LH induced prominent hypercalcemia in mice (26). We synthesized a new class of vitamin D₃ analogs [1,25(OH)₂-26,27-bishomo-19-nor vitamin D₃] in the in vitro biological activities of these analogs showed potent inhibition of proliferation of prostate, breast, and hematopoietic cancer cells and these effects were stronger than those of 1,25(OH)₂D₃. Although the antiproliferative effect of these analogs did not exceed that of the two analogs containing hexafluorines, including compound LH [1,25(OH)₂-26,27-hexafluoro-19-nor-D₃], they had much less calcemic activity. Taken together, 19-nor-26,27-bishomo analogs of vitamin D₃ may be a promising adjuvant in the treatment of a variety of cancers.

MATERIALS AND METHODS

Cell Lines. Cancer cell lines from the prostate (PC-3, DU 145, and LNCaP) and breast (MCF-7), as well as the myeloid leukemia cell line (HL-60), were obtained from American Type Culture Collection (Rockville, MD) and maintained according to their recommendations. All analogs used in this study were synthesized by Hoffmann LaRoche, Inc. The simplified code names and structures of the 1,25(OH)₂D₃ analogs are shown in Fig. 1. The vitamin D₃ compounds were dissolved in absolute ethanol at 10⁻⁷ mol/liter, stored at −20°C, and protected from light. The concentrations of the analogs were determined via UV absorbance using their molar extinction coefficient at 264 nmol/liter. For in vitro use, dilutions were made in the same tissue culture media as those used for cell
Fig. 1. Chemical structures of vitamin D₃ analogs used in this study.

Cell Cycle Analysis. MCF-7 cells (5 × 10⁵) were exposed to 10⁻⁷ M of either 1,25(OH)₂D₃, compound Ro 27-2014, or compound LH (Ro 25-6760) for 4 days in liquid culture. Total cells, both in suspension and adherent, were collected, washed, and stained with propidium iodide. The cells were adjusted to 1 × 10⁶ viable cells/ml and fixed in 2:1 ratio (vol/vol) in chilled methanol overnight before staining with propidium iodide (Promega), as described previously (30). Cell cycle status was analyzed on a Becton Dickinson Flow Cytometer and CellFIT Cell-Cycle Analysis software.

Serum Calcium Levels in Vivo. Thirty male BALB/c mice at 8–9 weeks of age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), maintained in pathogen-free conditions, and fed a standard laboratory diet. Mice were divided randomly into 6 groups of 5 mice: group A, control (PBS); group B, 1,25(OH)₂D₃ (compound C; 0.05 µg/mouse); group C, Ro 27-2014 (0.025 µg/mouse); group D, Ro 27-2014 (0.05 µg/mouse); group E, Ro 27-2014 (0.1 µg/mouse); group F, compound LH (0.05 µg/mouse). Five mice per group were injected i.p. three days a week (Monday, Wednesday, and Friday) with either vitamin D₃ compound or diluant (100 µl/mouse) for 5 weeks, as previously reported (26). Serum calcium levels were measured on...
days 8, 22, and 36 by DuPont Analyst Bencktop Chemistry System (Dade International, Newark, DE). Body weights were also measured every week.

RESULTS

Effect of Vitamin D3 Analogs on Clonal Proliferation of a Variety of Cancer Cell Lines. Prostate cancer (PC-3, LNCaP, and DU 145), breast cancer (MCF-7), and myeloid leukemia (HL-60) cells were cultured in soft agar in the presence of vitamin D3 analogs at $10^{-11}$ to $10^{-6}$ M. Colonies were counted, and dose-response curves were drawn (Fig. 2). The means of these dose-response curves were plotted on semi-logarithm graph paper, and the ED_{50} of colony formation was determined (Table 1). MCF-7, HL-60, and LNCaP cells were sensitive to the vitamin D3 analogs. PC-3 cells were slightly resistant, and DU 145 cells were resistant to these analogs. All vitamin D3 analogs potently inhibited clonal proliferation of MCF-7 and HL-60 cells, and 6 of 10 analogs achieved $\geq$50% inhibition of clonal growth of LNCaP cells. Only LT (Ro 25-9022) and LH (Ro 25-6760) vitamin D3 analogs inhibited $\geq$50% of the clonal growth of PC-3 cells. The 19-nor-hexafluoride analogs of vitamin D3 (compounds LH and LT) were more potent than any of the 19-nor-bishomo vitamin D3 analogs. Nevertheless, each of the 19-nor-26,27-bishomo-D3 analogs was more effective than compound C [1,25(OH)_2D_3]. The analog Ro 27-2014 was the most potent of the 19-nor-26,27-bishomo-D3 analogs. For example, it inhibited 50% of the proliferation of LNCaP cells at $6 \times 10^{-9}$ M, $3 \times 10^{-9}$ M, and $2 \times 10^{-10}$ M, respectively, which was about 10-fold more potent than 1,25(OH)_2D_3. The second most potent 19-nor-26,27-bishomo-D3 analog was Ro 27-2015, which was also about 10-fold more potent than 1,25(OH)_2D_3. The third most potent 19-nor-26,27-bishomo-D3 analog was Ro 27-2013. The structures of Ro 27-2015 and Ro 27-2013 are identical to compounds LH and LT, respectively, except that they have a bishomo instead of hexafluorine substitution at C-26,27.

Pulse-Exposure Experiments. To examine whether the clonogenic growth arrest of cancer cells by vitamin D3 analogs was irreversible, MCF-7 breast cancer cells were cultured in liquid medium with $10^{-7}$ M of vitamin D3 compounds [1,25(OH)_2D_3, Ro 27-2014, and LH] for various durations, thoroughly washed, and plated in soft agar in the absence of ligands. Colonies were counted on day 14. The mean time-response curve indicated that more than 50% of the clonogenic cells were inhibited by 4 days of exposure to compound Ro 27-2014, suggesting that it was capable of mediating an irreversible inhibition of growth of MCF-7 cells (Fig. 3). Results with compound LH showed 50% irreversible clonal inhibition by a 3-day pulse exposure. 1,25(OH)_2D_3 did not inhibit 50% clonal proliferation (peak 5 days; Fig. 3).

Cell Cycle Analyses. Analyses of the cell cycle of MCF-7 breast cancer cells after exposure to $10^{-7}$ M of vitamin D3 analogs [1,25(OH)_2D_3, Ro 27-2014, and LH] for 4 days showed that these cells had a dramatic increase in the percentage of cells in G_0-G_1 (Fig. 4; control, 49.9 $\pm$ 3.5%; 1,25(OH)_2D_3, 65.3 $\pm$ 4.3%; Ro 27-2014, 72.8 $\pm$ 8.9%; LH, 76.7 $\pm$ 3.0%). These cells showed a dramatic decrease in their S phase (control, 35.8 $\pm$ 3.5%; 1,25(OH)_2D_3, 18.6 $\pm$ 4.3%; Ro 27-2014, 13.1 $\pm$ 6.2%; LH, 9.3 $\pm$ 4.1%).

Serum Calcium Levels in Vivo. The major side effect of vitamin D3 analogs is hypercalcemia. Analysis of the calcemic potency is very relevant when choosing a suitable vitamin D3 compound for clinical trials. We compared the hypercalcemic effects of Ro 27-2014 with compound LH (hexafluorine-containing analog) and 1,25(OH)_2D_3 (compound C). Previous studies showed that mice who received 0.0625 $\mu$g of 1,25(OH)_2D_3 had very mild hypercalcemia, and mice that received greater than 0.0625 $\mu$g of compound LH had lethal hypercalcemia (25, 26). After a preliminary experiment, three doses of compound Ro 27-2014 (0.025 $\mu$g, 0.05 $\mu$g and 0.1 $\mu$g) were chosen and compared with 0.05 $\mu$g of 1,25(OH)_2D_3 and 0.05 $\mu$g of compound LH (Fig. 5).

After 5 weeks of study, all mice survived. Those that received 0.05 $\mu$g of compound C [1,25(OH)_2D_3] had serum Ca^{2+} levels of approximately 10 mg/dl, which was within the normal range (normal 8.5-10.5 mg/dl). The 19-nor-hexafluoride analog of vitamin D3 (compound LH, 0.05 $\mu$g) was very toxic, with serum Ca^{2+} levels of approximately 18 mg/dl. The mice receiving 0.025 and 0.05 $\mu$g of compound Ro 27-2014 had normal Ca^{2+} levels, and those receiving 0.1 $\mu$g of compound Ro 27-2014 had an elevated serum mean serum Ca^{2+} level of approximately 15 mg/dl, which was lower than that produced by 0.05 $\mu$g of compound LH. The experimental groups were weighed every week. The hypercalcemic mice had reduced weights [after the 5 week study: control, 27.9 $\pm$ 2.5 g; 1,25(OH)_2D_3, 27.0 $\pm$ 1.7 g; Ro 27-2014 (0.025 $\mu$g), 25.6 $\pm$ 1.9 g; Ro 27-2014 (0.05 $\mu$g), 25.2 $\pm$ 1.2 g; Ro 27-2014 (0.1 $\mu$g), 17.2 $\pm$ 1.1 g; LH, 17.1 $\pm$ 1.2 g].

DISCUSSION

Induction of differentiation and inhibition of proliferation through biological modifiers such as 1,25-vitamin D3 can provide a new therapeutic approach for various types of cancers (5-21). Development of vitamin D3 analogs that exert antiproliferative effects without producing hypercalcemia is critical for the successful application of this type of therapy. Previous studies showed that 1,25(OH)_2D_3 analogs that no longer had C-19 (19-nor-1,25D_3 analogs) were potent against human myeloid leukemia cell lines (31). Moreover, the addition of six fluorines on the side chain (C-26 and C-27) of the 19-nor-D_3 analogs enhanced their potency against prostate cancer (28), breast cancer (27), and leukemia (29). These 19-nor-hexafluoro analogs were 5-350-fold more potent than 1,25(OH)_2D_3, especially compound LH (Ro 25-6760), which was the most potent inhibitor of clonal proliferation of cancer cells. Also, the introduction of fluorine molecules to the end of the side chain enhanced the ability of vitamin D3 analogs to induce differentiation of HL-60 cells, presumably by a decrease in metabolic inactivation via the 26- and 27-hydroxylation pathway (24, 32, 33). Also, previous studies showed that the introduction of double and triple bonds at positions C-16 and C-23 was 2-4-fold more potent than 1,25(OH)_2D_3 (34, 35). However, 19-nor-26,27-F_6-D_3 analogs caused severe hypercalcemia when administered at pharmacologically active doses in vivo (26). Therefore, we synthesized 19-nor-26,27-bishomo-D_3 analogs by replacing the six fluorines with a bishomo modification, and we examined their biological effects on prostate, breast, and leukemia cell lines.

This study demonstrated that the six bishomo compounds and one trishomo analog of 1,25(OH)_2D_3 had remarkable abilities to inhibit the clonal proliferation of these cell lines. None of these analogs exceeded the potency of the 19-nor-hexafluoride analogs in clonogenic assay against cancer cells. The structures of Ro 27-2015 and Ro 27-2013 are identical to compounds LH and LT, respectively, but differ by their modifications at C-26,27. These findings indicate that the fluorine substitutions at C-26,27 enhanced the antiproliferative activity of the analog, as previously reported (32, 33). In addition, we have previously showed that the 20-epi-conformation of the 1,25(OH)_2D_3 [20-epi-1,25(OH)_2D_3] markedly enhanced the potency of vitamin D3 compounds (26, 30, 36). Therefore, we constructed two 19-nor-26,27-bishomo-D_3 analogs that also contained a 20-epi substitution: Ro 27-0256 and Ro 26-9737. These two analogs were synthesized by adding either 20-epi or 26a,27a-cyclo-20-epi substitution to Ro 27-2015. These were no more potent than Ro 27-2015. The structural benefit gained by the 20-epi-conformation appears to be lost by the addition of C-16-ene, as previously noted (28, 29). In this series
Fig. 2. Dose-response effects of vitamin D₃ compounds on clonal proliferation of PC-3, LNCaP, DU 145, MCF-7, and HL-60 cancer cells. Results are expressed as a mean percentage of control plates containing no vitamin D₃ compounds. Each data point represents a mean of at least three independent experiments with triplicate dishes. Bars, SD.
Table 1  Inhibition of clonal proliferation of tumor cells by vitamin D₃ analogs

<table>
<thead>
<tr>
<th>Analog</th>
<th>PC-3</th>
<th>LNCaP</th>
<th>DU145</th>
<th>MCF-7</th>
<th>HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)₂D₃</td>
<td>5 × 10⁻⁸</td>
<td>5 × 10⁻⁸</td>
<td>NR</td>
<td>3 × 10⁻⁸</td>
<td>3 × 10⁻⁹</td>
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<tr>
<td>LT</td>
<td>2 × 10⁻⁷</td>
<td>1 × 10⁻⁹</td>
<td>NR</td>
<td>6 × 10⁻¹⁰</td>
<td>2 × 10⁻¹⁰</td>
</tr>
<tr>
<td>LH</td>
<td>8 × 10⁻⁹</td>
<td>2 × 10⁻⁷</td>
<td>NR</td>
<td>6 × 10⁻¹⁰</td>
<td>8 × 10⁻¹¹</td>
</tr>
<tr>
<td>Ro 27-2014</td>
<td>NR</td>
<td>1 × 10⁻⁹</td>
<td>NR</td>
<td>3 × 10⁻⁹</td>
<td>2 × 10⁻⁹</td>
</tr>
<tr>
<td>Ro 27-2015</td>
<td>NR</td>
<td>2 × 10⁻⁷</td>
<td>NR</td>
<td>4 × 10⁻⁹</td>
<td>3 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Ro 27-2735</td>
<td>NR</td>
<td>1 × 10⁻⁶</td>
<td>NR</td>
<td>5 × 10⁻⁹</td>
<td>4 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Ro 27-2735</td>
<td>NR</td>
<td>1 × 10⁻⁹</td>
<td>NR</td>
<td>2 × 10⁻⁸</td>
<td>3 × 10⁻⁹</td>
</tr>
<tr>
<td>Ro 26-9737</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>1 × 10⁻⁸</td>
<td>2 × 10⁻⁹</td>
</tr>
<tr>
<td>Ro 27-0256</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>2 × 10⁻⁸</td>
<td>2 × 10⁻⁹</td>
</tr>
<tr>
<td>Ro 25-8584</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>2 × 10⁻⁸</td>
<td>3 × 10⁻⁹</td>
</tr>
</tbody>
</table>

* NR, ED₅₀ was not reached at ≤10⁻⁸ M analogs.

Dose-response experiments were performed; the data were plotted on semilogarithm graphs (Fig. 2), and the curves were used to calculate the concentration of the analogs achieving a 50% inhibition (ED₅₀) of clonal growth.

Inhibition of clonal proliferation, ED₅₀ (m)

Tumor cell lines

<table>
<thead>
<tr>
<th>Analogs</th>
<th>Inhibition of clonal proliferation, ED₅₀ (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td></td>
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<tr>
<td>LNCaP</td>
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<td>DU145</td>
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<td>MCF-7</td>
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<td>HL-60</td>
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</table>

of new 19-nor-26,27-bishomo-D₃ analogs, the most potent analog was Ro 27-2014 (ED₅₀: LNCaP, 6 × 10⁻⁹ m; MCF-7, 3 × 10⁻⁹ m; HL-60, 2 × 10⁻¹⁰ m); it was approximately 10-fold more potent than 1,25(OH)₂D₃, therefore, we focused on the scope of activity of this analog, comparing it to 1,25(OH)₂D₃ and compound LH.

With respect to pulse exposure of MCF-7 breast cancer cells, 5 days of exposure to analog Ro 27-2014 (10⁻⁷ m), washing, plating in soft agar, and enumerating colonies 14 days after plating resulted in 50% inhibition of colony formation, whereas 3 days of exposure to analog LH (10⁻⁷ m) resulted in a 50% decrease in colony formation. Although results with 1,25(OH)₂D₃ (compound C, 10⁻⁷ m, ≤5 days) paralleled results with Ro 27-2014 and LH, 1,25(OH)₂D₃ did not achieve a 50% inhibition of colony formation. These results indicated that these compounds inhibited growth of MCF-7 cells by a mechanism other than one that is merely cytostatic.

The mechanism by which the vitamin D₃ compounds mediate their antiproliferative effects remains unknown. One effect observed in cancer cells exposed to 1,25(OH)₂D₃ is accumulation of cells in G₁. Our cell cycle analyses revealed prominent G₁ arrest of MCF-7 cells after their exposure to analog Ro 27-2014 as well as compound LH, which were both more potent than 1,25(OH)₂D₃. Many factors can lead to cell cycle arrest, but the cyclin-dependent kinase inhibitors known as p21waf1 and p27kip1 play a central role in this process. These proteins are associated with the G₁ arrest that occurred after the cells were treated with vitamin D₃ (37). Although we did not examine the effect of Ro 27-2014 on levels of p21waf1 and p27kip1, we previously have shown that compound LH induced increased expression of these cyclin-dependent kinase inhibitors (27, 29).

Calcium studies in mice found that Ro 27-2014 produced less hypercalcemia than the 19-nor-26,27-F₆-D₃ analog LH. The serum calcium levels of the mice receiving Ro 27-2014 were almost identical to those of mice receiving 1,25(OH)₂D₃. Compared with 1,25(OH)₂D₃, Ro 27-2014 was almost 10-fold more potent in its ability to inhibit clonal proliferation of cancer cells. In contrast, the addition of fluorines at C-26,27 (compound LH) enhanced not only the ability of vitamin D₃ analogs to inhibit clonal proliferation of cancer cells but also to produce the hypercalcemia, thus resulting in a small therapeutic index. In summary, we have synthesized several vitamin D₃ analogs that have the addition of two or three extra carbons to side chain, desaturation of their side chain and C-16, and removal of C-19; these compounds have enhanced abilities to inhibit growth of several types of cancer cells without causing hypercalcemia. Further testing using animal models in the setting of low tumor burden may result in a new therapeutic approach.
Calcium study in balb/c mice

Fig. 5. The effect of vitamin D analogs on serum calcium in mice. Each data point represents the mean ± SD of five mice. The mice received i.p. injections of vitamin D compound three days a week (Monday, Wednesday, and Friday).

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19-nor-26,27-bishomo-Vitamin D₃ Analogs: A Unique Class of Potent Inhibitors of Proliferation of Prostate, Breast, and Hematopoietic Cancer Cells

Tetsuya Kubota, Kozo Koshizuka, Michiaki Koike, et al.


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