19-nor-Hexafluoride Analogue of Vitamin D₃: A Novel Class of Potent Inhibitors of Proliferation of Human Breast Cells¹

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ABSTRACT

Breast cancer cells express vitamin D₃ receptors and 1,25-dihydroxy-vitamin D₃ suppressed growth of these cells. We have synthesized six novel vitamin D₃ analogues to identify those with expanded capacity to inhibit the proliferative ability of breast cancer cells. These analogues incorporated many of the structural motifs shown previously to have antiproliferative activity in several cell types. Six breast cancer cell lines were used as targets. Dose-response studies showed that each of the analogues had antiproliferative activities, and LH [1,25(OH)₂]-16-ene-23-yne-26,27-F₃, 19-nor D₃] was the most potent analogue, suppressing at 10⁻¹¹ M greater than 50% clonal proliferation (ED₅₀) of the MCF-7 and SK-BR-3 breast cancer cells, increasing the proportion of MCF-7 cells in the Gₛ-G₁ phase, and decreasing those in the S phase of the cell cycle. Pulse-exposure studies showed that a 3-day exposure to LH (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 analogue, suggesting that the growth inhibition mediated by LH is irreversible. The cyclin-dependent kinase inhibitor known as p27Kip1 helps regulate the cell cycle and can mediate growth arrest in response to extracellular growth inhibitors. The analogue LH (10⁻⁷ M) induced elevated expression of p27Kip1 in MCF-7 and SK-BR-3 cells. Taken together, these results indicate that LH is an extremely potent vitamin D₃ analogue markedly inhibiting clonal growth of MCF-7 and SK-BR-3 cells with concomitant cell cycle arrest at Gₛ-G₁ and increased expression of p27Kip1. Compound LH is worthy of in vivo analysis for possible future clinical trials.

INTRODUCTION

Breast cancer is the most common malignant disease of women in the United States. Improvement in hormonal and cytotoxic therapies have not led to either a major lengthening of remissions or increase in cures in advanced breast cancer. Antiestrogens have provided the most effective endocrine therapy for advanced breast cancer (1). The 1,25 D₃ is a member of the secosteroid family. Most breast cancer cell lines and more than 80% of breast tumors expressed high affinity VDRs (2–4). Reminiscent of estrogen receptor data, patients with primary carcinoma of the breast who were VDR positive had significantly longer disease-free survival than those with VDR-negative tumors (5). The 1,25 D₃ and its analogues inhibit proliferation of breast cancer cells in vitro (6–8). Likewise, 1,25 D₃ and its related analogues decreased the progression of breast cancer and other carcinomas in vivo (9–11), inhibited metastatic spread of tumors cells (12–14), and promoted differentiation of breast cancer cells as well as other varieties of cancer (5, 8, 15–18). However, thecalcemic side effects of 1,25 D₃ have prevented its application as a pharmaceutical agent. Synthesis of analogues of 1,25 D₃ with potent antiproliferative and differentiation activity against cancer cells with decreased risk of inducing hypercalcemia has been reported (9, 11, 12, 19–21).

Previously, we have studied the in vitro biological activities and mechanism of action of four potent 1,25 D₃ analogues (KH1060, 20-epi-1,25(OH)₂-D₃, 1,25(OH)₂-16-ene-D₃, and V) on the proliferation and differentiation of six breast cancer cell lines. In that study, KH1060 was the most potent 1,25 D₃ analogue inhibiting clonal growth of four breast cancer lines, with decreased bcl-2 and cell cycle arrest at Gₛ-G₁ (22).

p27Kip1 is a recently cloned cyclin-dependent kinase inhibitor associated with arrest of the cell cycle (23, 24). We have examined for p27Kip1 mutations in 36 primary breast cancers and 9 breast cancer cell lines (25). Only two point mutations were found in the primary tumors. Additional studies showed that overexpressed Kip proteins caused cell cycle arrest, and expression of p27Kip1 was up-regulated by exposure of the cells to several antimitotics (23, 26–30). In this study, we have shown that LH was the most potent of a new series of 1,25 D₃ analogues in mediating the inhibition of clonal growth of MCF-7 and SK-BR-3 breast cancer cells associated with cell cycle arrest at Gₛ-G₁ and increased expression of p27Kip1.

MATERIALS AND METHODS

Cell Lines. The breast cancer cell lines (MDA-MB-436, MCF-7, SK-BR-3, BT-474, BT-20, and MDA-MB-231) were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM or McCoy’s media (Life Technologies, Inc., Grand Island, NY) containing 10% bovine fetal serum, according to the recommendations of American Type Culture Collection, in culture flasks with vented filter caps (Costar, Cambridge, MA).

Vitamin D₃ Compounds. The vitamin D₃ compounds were dissolved in absolute ethanol at 10⁻¹⁷ M as stock solution, which were stored at –20°C and protected from light. The analogues C, Y, LH, KS, KY, LW, and LA were synthesized by Hoffmann LaRoche, Inc. (Fig. 1).

Clonogenic Assay in Soft Agar. Breast cancer cells were cultured in a two-layer soft agar system for 14 days, as described previously (31).

Pulse-Exposure Experiments. The MCF-7 cells were exposed to analogue LH (10⁻⁹ M) for various durations. After incubation, the cells were carefully washed twice, counted, and plated into 24-well plates for soft agar colony assay.

Cell Cycle Analysis by Flow Cytometry. Cell cycle analysis was performed on breast cancer cells incubated for 72 h with or without LH at 10⁻⁷ M. The melanin-fixed cells were incubated for 30 min at 4°C in the dark with a solution of 50 μg/ml propidium iodide, 1 mg/ml RNase (100 units/ml; Sigma Chemical Co.), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CELLFit program (Becton Dickinson), whereby the S phase was calculated with a Rfit model.

Western Blot Analysis. Cultured cells were washed twice with PBS and then lysed in 1 ml/10⁵ cells of 50 mM Tris (pH 8.0). 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin for 30 min at 0°C. Insoluble material was removed by centrifugation at 14,000 rpm at 4°C.

Received 3/21/97; accepted 8/15/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This study was supported by NIH Grants CA42377, CA42710, CA7067501-01, and CA26038; United States Army Grant DAMD17-96-1-0054; and grants from the Concern Foundation and Parker Hughes Trust.

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VITAMIN D₃ ANALOGUES EFFECTIVE AGAINST BREAST CANCER

Fig. 1. Structures and code names of the novel vitamin D₃ analogues examined in this study.

RESULTS

Effect of Vitamin D₃ Analogues on Clonal Proliferation of Breast Cancer Cell Lines. Breast cancer cells were cloned in soft agar in the presence of vitamin D₃ analogues at 10⁻¹¹ to 10⁻⁷ M. Dose-response curves were drawn (Fig. 2), and the effective dose that inhibited 50% colony formation (ED₅₀) was determined (Table 1). The 1,25 D₃ analogues were effective in inhibition of clonal proliferation of two of the six breast cancer cell lines (MCF-7 and SK-BR-3; Fig. 2, a and b).

The BT-474 and MDA-MB-231 breast cancer cells were fairly resistant to the vitamin D₃ analogues, and the MDA-MB-436 and BT-20 were even more resistant (Fig. 2, c–f). For the two sensitive breast cancer cell lines (Fig. 2, a and b) as well as MDA-MB-231, the LH analogue was the most potent compound. The LH analogue achieved an ED₅₀ of 8 x 10⁻¹¹ and 8 x 10⁻¹⁰ M for SK-BR-3 and MCF-7, respectively (Table 1).

Pulse-Exposure Experiments. The MCF-7 cells were exposed to the analogue LH (10⁻⁷ M) for various durations, washed three times to remove the analogue, and plated in soft agar; and colony number was enumerated on day 14 (Fig. 3). Fifty % of the clonogenic cells were inhibited by 3 days of exposure to analogue LH, suggesting that this compound was capable of mediating an irreversible inhibition of the growth of these cells.

Cell Cycle Analysis. Effect of LH on the cell cycle of breast cancer cells was determined by studying the MCF-7 cells. These cells had a significant increase in the number of cells in the G₀-G₁ phase of the cell cycle [70 ± 2% with LH (10⁻⁷ M) for 72 h, 52 ± 1.7% in for 10 min. Protein concentrations were determined using a Bio-Rad kit. Proteins (40 µg) were size fractionated under denaturing conditions on 12.5% SDS-running gel and transferred to Millipore membrane and exposed without drying to the X-ray film overnight. The p27kip1-specific band was detected by Western blot hybridization of the membrane with purified anti-p27kip1 antibody and detection of the signal was with the ECL system (Amersham). A rabbit polyclonal anti-serum specific for human p27kip1 (Santa Cruz) was used for Western blot analysis.
VITAMIN D<sub>3</sub> ANALOGUES EFFECTIVE AGAINST BREAST CANCER

Fig. 2. Dose-response effects of vitamin D<sub>3</sub> compounds on clonal proliferation of breast cancer cell lines. Results are expressed as a mean percentage of control plates containing no vitamin D<sub>3</sub> compounds (means of at least three experiments with triplicate dishes; bars, SD).

control cells) with a concomitant decrease in the S phase [16 ± 2% with LH (10<sup>-7</sup> M) for 72 h, 35 ± 2.4% in control cells] (P < 0.05; Fig. 4).

Increased Levels of p27<sup>Kip1</sup> Induced in MCF-7 and SK-BR-3 Cell Lines during Exposure to Analogue LH. The MCF-7 and SK-BR-3 cells had a moderate level of expression of p27<sup>Kip1</sup> (Fig. 5, Lanes 2 and 4) as determined by Western blot analysis. Exposure of these cells to LH (10<sup>-7</sup> M) resulted in a modest increase in expression of p27<sup>Kip1</sup> with levels increasing 40% in SK-BR-3 cell line at day 3 of exposure and 30% in MCF-7 cells at day 6 of exposure to the analogue (Fig. 5). Similar findings were found on repeat experiments.
VITAMIN D3 ANALOGUES EFFECTIVE AGAINST BREAST CANCER

Table 1  Inhibition of clonal proliferation of breast cancer cell lines by vitamin D3 analogues

<table>
<thead>
<tr>
<th>Breast cancer cell lines</th>
<th>1,25 D3</th>
<th>LA</th>
<th>LH</th>
<th>KS</th>
<th>KY</th>
<th>KW</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>3 × 10^-8</td>
<td>1 × 10^-8</td>
<td>8 × 10^-10</td>
<td>3 × 10^-8</td>
<td>8 × 10^-9</td>
<td>1 × 10^-8</td>
<td>5 × 10^-10</td>
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<tr>
<td>SK-BR-3</td>
<td>8 × 10^-9</td>
<td>3 × 10^-9</td>
<td>8 × 10^-11</td>
<td>8 × 10^-11</td>
<td>8 × 10^-10</td>
<td>6 × 10^-11</td>
<td>8 × 10^-10</td>
</tr>
<tr>
<td>BT-474</td>
<td>N.R.</td>
<td>N.R.</td>
<td>8 × 10^-10</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
</tr>
</tbody>
</table>

*a Dose-response curves (Fig. 2) were used to calculate the concentration of the analogues achieving a 50% inhibition (ED50) of clonal growth.

*b N.R., the ED50 was not reached at ≤10^-7 M of the 1,25 D3 analogue.

**DISCUSSION**

The six breast cancer cell lines used in our study varied in their sensitivities to the clonal inhibitory effects of the various 1,25 D3 compounds. The most sensitive lines, MCF-7 and SK-BR-3, were inhibited by each of the analogues. In contrast, MDA-MB-436, BT-20, BT-474, and MDA-MB-231 cells were entirely or partially resistant to the 1,25 D3 analogues used in this study, suggesting that preclinical testing of the breast cancer cells in vitro may be helpful in the selection of patients for clinical trials with these analogues.

The LH was the most active analogue in this study, which has double bonds at C16, triple bonds at C23, six fluorine substitutions at C26 and C27, and removal of a methylene at C19. Previously, we have shown that hexafluoro analogues of 1,25-(OH)2D3 were 5- to 350-fold more potent than 1,25 D3 as antileukemic agents (21). LH also was very active against prostate cancer cell lines (LNCaP, PC-3, and DU-145 cell lines; Ref. 32).

Earlier, we have shown that a 20-epi-vitamin D3 (KH-1060) was a very potent analogue against breast cancer cell lines (22). We synthesized a 20-epi-analogue (compound KY) that combined structural motifs shown previously to be important for antiproliferative activity against cancer cells (C16 and C23 double bonds, C26 and C27 hexa-fluorines). But, the addition of these structural elements to the 20-epi backbone did not increase antiproliferative activity (Fig. 2) as compared to the 20-epi form alone (22).

One of the interesting biologically and potentially clinically relevant observations that was made in this and another study done by us recently (33) is that cancer cells of different tissues can display different sensitivities to the same vitamin D3 analogues. For example, analogue LA is one of the most potent analogues in its inhibition of clonal growth of HL60 leukemia cells, with an ED50 of 2 × 10^-11 m, whereas analogue LH had an ED50 of 2 × 10^-10 m in the same assay. In contrast, analogue LA was 1 to 2 logs less effective than analogue LH in its antiproliferative activity against breast and prostate cancer cell lines (Table 1; Refs. 32 and 33). These results suggest that the mechanism of growth inhibition of cancer cells by vitamin D3 analogues may vary between different types of tissues.

Pulse-exposure of MCF-7 cells for 3 days to analogue LH (10^-7 m), washing, plating in soft agar, and enumerating colony formation 14 days after plating resulted in 50% inhibition of colony formation. These results suggest that LH inhibited growth of breast cancer cells by a mechanism other than one that is merely cytostatic. Furthermore, LH increased the number of MCF-7 cells in G1 and decreased the number in S phase (Fig. 4).

Recently, several CDK inhibitor (CDK1) genes have been cloned, and they have been classified into two groups (34). One group includes the INK4 proteins: p16INK4A (35, 36); p15INK4B (36, 37-39); p18INK4C; and p19INK4D (39). These specifically inhibit the CDK complexes involving CDK4 and CDK6, which are CDKs expressed exclusively at the mid-G1 phase of the cell cycle (34, 37, 39). The other group consists of p21Waf1 (40-45), p27Kip1 (23, 24), and p57Kip2 (46, 47). These proteins are structurally and functionally unrelated to the INK4 genes. The p27Kip1 protein has 42% amino acid homology with p21Waf1 and 47% similarity with the p57Kip2 protein within the NH2-terminal domain, which mediates the inhibition of CDK (23, 24, 46-49). In comparison to the INK4 proteins, the
**ACKNOWLEDGMENTS**

We thank Peggy Palmer for excellent secretarial help.

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