Growth Inhibition of HT-29 Human Colon Cancer Cells by Analogues of 1,25-Dihydroxyvitamin D₃


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ABSTRACT

The use of 1,25-dihydroxyvitamin D₃ as an antiproliferative agent in the treatment of cancer is limited by its hypercalcemic effects. Analogues with equivalent or greater antiproliferative activities but smaller hypercalcemic effects have been developed. The antiproliferative effects of 1,25-dihydroxyvitamin D₃ and four analogues were studied in HT-29 and SW620 human colon cancer cells, moderate and low expressors of the vitamin D receptor, respectively. HT-29 is a primary, moderately differentiated, cell line, while SW620 is metastatic and poorly differentiated. Growth curve studies, proliferation assays, and clonogenic assays were used to assess the antiproliferative effects of 1,25-dihydroxyvitamin D₃, 1,25-dihydroxy-16-ene-23-yne-D₃, 1,25-dihydroxy-16,23E-diene-26,27-hexafluoro-D₃, and 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-D₃. Growth of HT-29 cells was significantly inhibited by all four analogues at 10⁻⁶ M (P < 0.05). Analogues 1,25-dihydroxy-26,27-hexafluoro-16-ene-23-yne-D₃, 1,25-dihydroxy-16,23E-diene-26,27-hexafluoro-D₃, and 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-D₃ were 2 times as potent as analogue 1,25-dihydroxy-16-ene-23-yne-D₃, and 1,25-dihydroxyvitamin D₃. SW620 cells did not show any growth inhibition with any of the compounds tested. The affinities of the three most potent analogues for the vitamin D receptor were similar to that of 1,25-dihydroxyvitamin D₃, while that of analogue 1,25-dihydroxy-16-ene-23-yne-D₃ was lower. These results demonstrate that, as in leukemic cells, analogues of 1,25-dihydroxyvitamin D₃ are potent antiproliferative agents in colon cancer cells and this activity is most likely mediated through the vitamin D receptor.

INTRODUCTION

1,25-Dihydroxyvitamin D₃ and related compounds appear to be involved in cell growth and differentiation, and this may play a significant role in the treatment of cancer (1). This function is independent of the role of 1,25-dihydroxyvitamin D₃ in calcium homeostasis. In 1981, Abe et al. (2, 3) noted that 1,25-dihydroxyvitamin D₃, the active form of vitamin D, induced the differentiation of HL-60 and M1 leukemia cells into macrophages. 1,25-Dihydroxyvitamin D₃ has been shown to have antiproliferative effects on osteosarcoma, breast carcinoma, and colon carcinoma cell lines (4, 5). These effects are mediated by the binding of 1,25-dihydroxyvitamin D₃ to a specific intracellular receptor (6, 7). The VDR, ² which is a member of the steroid hormone receptor superfamily, regulates gene transcription through interaction with hormone response elements in the promoter region of target genes (8). VDR has been demonstrated in melanoma, osteosarcoma, and breast, lung, ovary, and colon carcinoma cell lines (6). Our studies in colon adenoma and adenocarcinoma cells have shown a general correlation between the level of VDR and the degree of differentiation of the cell line (9). The cells with higher levels of vitamin D receptor were more responsive to the antiproliferative effects of 1,25-dihydroxyvitamin D₃.

In vivo studies involving mice inoculated with leukemic cells showed prolonged survival after administration of 1,25-dihydroxyvitamin D₃ (2, 10). However, in humans, the hypercalcemic activity of 1,25-dihydroxyvitamin D₃ has prevented the achievement of adequate serum levels associated with antiproliferative effects (11). In clinical trials, after administration of 1,25-dihydroxyvitamin D₃ to preleukemic patients, hypercalcemia developed at a 1,25-dihydroxyvitamin D₃ serum concentration of 2 × 10⁻¹⁰ M. This concentration has not been shown to have differentiating or antiproliferative effects. Hence, investigators have developed analogues of vitamin D that retain the ability to induce differentiation and inhibit proliferation but cause a lower degree of hypercalcemia (12-17). These analogues, which are synthesized by modification of the side chain and the D-ring of 1,25-dihydroxyvitamin D₃ (12), have been analyzed mostly in leukemic cells. In this study, we examine the effects of four analogues of 1,25-dihydroxyvitamin D₃, i.e., 1,25-dihydroxy-16-ene-23-yne-D₃, 1,25-dihydroxy-26,27-hexafluoro-16-ene-23-yne-D₃, 1,25-dihydroxy-16,23E-diene-26,27-hexafluoro-D₃, and 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-D₃, on the proliferation of a VDR-positive human colon cancer cell line, HT-29, and a VDR-negative cell line, SW620.

MATERIALS AND METHODS

Cell Culture

HT-29 and SW620 human colon cancer cell lines available from the American Type Culture Collection (Rockville, MD) were grown as monolayers in RPMI 1640 medium (obtained from Biofluids, Rockville, MD) supplemented with 5% fetal calf serum, 2 mM glutamine, and 10 units/ml penicillin.

Reagents

1,25-Dihydroxyvitamin D₃ (vitamin D₃) and four synthetic vitamin D analogues, i.e., 1,25-dihydroxy-16-ene-23-yne-D₃, 1,25-dihydroxy-16,23E-diene-26,27-hexafluoro-D₃, and 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-D₃, were synthesized by Hoffmann-La Roche Laboratories (Nutley, NJ). They were all dissolved in absolute ethanol at 10⁻³ M concentration and kept at -20°C in glass containers protected from light. Concentrations were verified by UV absorption spectrophotometry (Beckman DU-65 spectrophotometer) at 265 nm, using a molar extinction coefficient of 17,000.

Stock solutions were diluted in control medium, which consisted of IMEM with 5% charcoal-stripped fetal calf serum, 2 mM glutamine, 10 units/ml penicillin, and 10 ng/ml insulin. The concentration of ethanol at 0.1% did not influence cellular growth or differentiation. The level of 1,25-dihydroxyvitamin D₃ in the control medium was measured to be 2.4 × 10⁻¹³ M (using an extraction and purification sequestration-saturation assay kit from Incstar Corp., Minneapolis, MN).

Cell Growth Experiments

Growth Curve Studies. Dose-response growth curve studies were performed with 1,25-dihydroxyvitamin D₃ and two of the analogues in HT-29 cells, which had previously been shown to be grown inhibited by 1,25-

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2 The abbreviations used are: VDR, vitamin D receptor(s); CEA, carcinoembryonic antigen; RCI, relative competitive index; 1,25(OH)₂-16-ene-23-yne-D₃; 1,25(OH)₂-26,27-F-16-ene-23-yne-D₃; 1,25-dihydroxy-26,27-hexafluoro-16-ene-23-yne-D₃; 1,25(OH)₂-16,23E-diene-26,27-F-D₃; 1,25-dihydroxy-16,23E-diene-26,27-hexafluoro-D₃; IMEM, Iscove’s minimum essential medium.
dihydroxyvitamin D₃ (9). The purpose of these studies was to determine the concentration at which significant growth inhibition could be demonstrated for these compounds. HT-29 monolayer cells (1 x 10⁵ cells/well) were plated in 6-well tissue culture plates. After overnight growth, experimental medium was added. This consisted of 1,25-dihydroxyvitamin D₃, analogue 1,25(OH)₂-16-ene-23-yne-D₃, or analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ at concentrations ranging from 10⁻¹² to 10⁻⁸ M. Control medium consisted of IMEM with 5% charcoal-stripped fetal calf serum. Subsequently, fresh treatment medium was added every other day. On days 2, 4, and 6 after the addition of treatment medium, cells were trypsinized and counted using a hemocytometer. Cell counts on day 6 were plotted as percentage of control.

In the next phase of the study, HT-29 and SW620 monolayer cells were plated in a similar fashion. After overnight growth, experimental medium, which consisted of control medium or medium containing 1,25-dihydroxyvitamin D₃ or analogues 1,25(OH)₂-16-ene-23-yne-D₃, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, or 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ (10⁻⁸ M), was added. Subsequently, fresh treatment medium was added every other day. The concentration of 10⁻⁸ M was chosen based on similar in vitro studies in leukemia cells and the results of the dose-response studies described above (13, 14). The antiproliferative potency of 1,25-dihydroxyvitamin D₃ and the four analogues was compared at this concentration based on the dose-response curves, which showed the agents to have potent antiproliferative effects at 10⁻⁸ M. Cell counts were done on days 2, 4, and 6 as described above. All counts were obtained in duplicate at each time point. The cell counts from day 6 were plotted as percentage of control. Error bars represent 1 SD of the mean. Student’s t-test was used to compare cell counts on day 6 of treatment.

Proliferation Assay. For each cell line, 2 x 10⁴ cells/well were plated in 24-well tissue culture plates (Costar, Cambridge, MA). After overnight growth, treatment medium, i.e., control medium or medium containing 1,25-dihydroxyvitamin D₃ or analogues 1,25(OH)₂-16-ene-23-yne-D₃, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, or 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ at a concentration of 10⁻¹⁰ M, was added. Forty-eight h later, fresh treatment medium was added. Ninety-six h after incubation with treatment medium, [³H]thymidine incorporation into DNA was measured. Cells were incubated with [³H]thymidine (10 µCi/ml) for 2 h at 37°C. After the cells were washed with phosphate-buffered saline, they were trypsinized, lysed by sonication, and incubated with 10% trichloroacetic acid for 15 min at 4°C to remove excess thymidine. The cell lysates were transferred to 0.45-µm-pore filters (Millipore, Bedford, MA). The filters were rinsed with 10% trichloroacetic acid. They were then placed in scintillation vials containing Aquasol-2 universal liquid scintillation cocktail. Radioactivity was counted with a Beckman LS 7500 scintillation counter. Thymidine incorporation was plotted as percentage of control. Error bars represent 1 SD of the mean (n = 3). Results were compared using Student’s t-test.

Soft Agar Assay. HT-29 and SW620 cells (2 x 10⁴) were plated in 35-mm tissue culture dishes in IMEM with 5% charcoal-stripped fetal calf serum and 2 mM glutamine agar (2.4%) added. After overnight growth, the cells were overlaid with experimental medium as described for the proliferation assay. The experiment was done in triplicate and the SD was calculated. After 20 days of incubation at 37°C, colonies were counted using a computerized image analyzer (Omnicon 3600 Image Analysis System) with a size cutoff of 60 µm. Colony counts were performed in triplicate and the SD was calculated. Counts were compared using Student’s t-test.

Determination Assays

HT-29 and SW620 cells were plated in 162-cm² tissue culture flasks. When 80% confluence was achieved, treatment medium, consisting of control medium or medium containing 1,25-dihydroxyvitamin D₃ or analogues 1,25(OH)₂-16-ene-23-yne-D₃ or 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ at a concentration of 10⁻¹⁰ M, was added to the flasks. Sodium butyrate (2 mM), a known differentiating agent in colon cancer cells, was used as a positive control. After an incubation period of 72 h, cells were harvested and lysed by sonication. Protein content was measured with a colorimetric assay (Bio-Rad). Mean absorbance was measured at 595 nm using a Beckman DU-65 spectrophotometer.

Alkaline Phosphatase Assay. Fifty-µg aliquots of each sample were measured and volumes were equilibrated to 50 µl. Standard solutions ranging from 0.2 ml hydroxyapatite slurry (50%, v/v, in 10 mM Tris) was added. The assay was done using the Roche CEA reagent set (item 43348). Each sample was done in duplicate and SD was calculated.

Vitamin D Receptor Competitive Binding Assay

The affinity of binding of analogues 1,25(OH)₂-16-ene-23-yne-D₃, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ was compared to that of [³H]-1,25-dihydroxyvitamin D₃ using the hydroxyapatite batch assay (18) and was expressed as RCI, with 1,25-dihydroxyvitamin D₃ arbitrarily being assigned a RCI of 100%. HT-29 cells were grown to 70% confluence in 162-mm² flasks. The medium was changed to IMEM with 5% charcoal-stripped fetal calf serum 1 day before the assay was performed. Cells were harvested by scraping with a rubber policeman and were rinsed three times with cold phosphate-buffered saline. They were then pelleted and resuspended in hypotonic buffer (0.3 M KCl, 10 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate, pH 7.4). After sonication, the suspension of cells was centrifuged at low speed (2500 x g at 4°C for 15 min). The nuclear pellet was discarded and the supernatant was used to high speed centrifugation (100,000 x g at 4°C for 60 min). The upper floating layer containing lipids was removed from the cytosolic fraction. Protein content was then adjusted to 1.3–1.5 mg/ml. A range of increasing concentrations of 1,25-dihydroxyvitamin D₃ and the four analogues (10⁻¹¹ to 10⁻⁸ M) was added to 10⁻¹⁰ M [³H]-1,25-dihydroxyvitamin D₃ (specific activity, 165 Ci/mmol) and incubated with 200 µl of cytosol at 16 h at 4°C. Incubation was terminated by transferring the tubes to ice, where 0.2 ml hydroxyapatite slurry (50%, v/v, in 10 mM Tris) was added to each. After a 15-min incubation, hydroxyapatite pellets were rinsed 3 times with 10 mM Tris solution containing 0.5% (v/v) Triton X-100. Radioactivity was extracted with 0.7 ml of 100% ethanol. After centrifugation, the supernatant was added to scintillation vials and allowed to evaporate overnight. Beckman Readysafe scintillation fluid was then added to each and radioactivity was measured. The relative competitive index for the four analogues was derived from a plot of the reciprocal of percentage maximum bound [³H]-1,25-dihydroxyvitamin D₃ as a function of the ratio of the concentration of competitor to [³H]-1,25-dihydroxyvitamin D₃. Each point represents the average of two determinations. The slope of the line obtained for each analogue divided by the slope of the line for 1,25-dihydroxyvitamin D₃ and multiplied by 100% yielded the respective RCI values. The slope was derived from a regression line using all points on the graph (the data points were fitted to a linear regression using least-squares fitting). Error bars represent 1 SD of the mean (n = 2).

RESULTS

Cell Growth Experiments

Growth Curve Studies. Fig. 1 shows the result of the dose-response growth curve studies in HT-29 cells. 1,25-Dihydroxyvitamin D₃, analogue 1,25(OH)₂-16-ene-23-yne-D₃, and analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ demonstrated a dose-dependent increase in their antiproliferative effects, with the highest degree of inhibition being seen at 10⁻⁸ M. Significant growth inhibition was demonstrated at 10⁻⁸ M (P < 0.05) with analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃. In view of the significant potency, this concentration was used in all subsequent studies.

In HT-29 cells, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ caused significant (P < 0.05) growth inhibition on day 6 (Fig. 2). Cell counts were reduced by 68.3%, 74.5%, and 69%, respectively, 1,25-
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Dihydroxyvitamin D₃ and analogue 1,25(OH)₂-16-ene-23-yne-D₃ suppressed cell growth by 33% and 38%, respectively (P > 0.05). In SW620 cells, no growth inhibition was seen with any of these compounds (Fig. 3).

**Proliferation Assay.** Compared to control, the growth of HT-29 cells, a moderate expressor of VDR, was inhibited by 34% after treatment with 10⁻⁸ M 1,25-dihydroxyvitamin D₃ (Fig. 4). In the same cell line, analogues 1,25(OH)₂-16-ene-23-yne-D₃ and 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ at the concentration of 10⁻⁸ M caused 45% and 46% growth inhibition, while treatment with analogues 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃ and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ produced maximal growth inhibition of 73% and 82%, respectively. The growth inhibitions shown by all four analogues were statistically significant (P < 0.05). Neither 1,25-dihydroxyvitamin D₃ nor any of the four synthetic analogues caused any significant growth inhibition in SW620 cells, a low VDR expressor.

**Soft Agar Assay.** The most significant growth inhibition in HT-29 colon cancer cells was seen with analogues 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃ and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃, which inhibited clonogenic growth by 70.1% and 74.2%, respectively (P < 0.05)(Fig. 5). Analogue 1,25(OH)₂-16-ene-23-yne-D₃ reduced growth by 41.4% and 1,25-dihydroxyvitamin D₃ by 21% (P < 0.05). 1,25-Dihydroxyvitamin D₃ and the two synthetic analogues did not increase CEA production in SW620 cells either. CEA levels were lower in SW620 cells than in HT-29 cells, with levels of 30–40 ng/mg of protein.

**Vitamin D Receptor Competitive Binding Assay**

In determining the RCI of analogues 1,25(OH)₂-16-ene-23-yne-D₃, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃, the RCI for 1,25-dihydroxyvitamin D₃ was arbitrarily set at 100% (Fig. 7). The affinities of the analogues for the vitamin D receptor was 59%, 95%, 93%, and 100%, respectively (Table 1).
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**HT29 Growth Curve Study**

**Fig. 2.** Growth curve studies in HT-29 human colon cancer cells, assessing the effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and analogues 1,25(OH)₂-16-ene-23-yne-D₃, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃, at 10⁻⁵ M, on cell counts. Cell counts on day 6 are expressed as percentage of control. On day 6, significant (P < 0.05) growth inhibition was seen in HT-29 cells with analogues 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ (*).

**DISCUSSION**

In this study, we have compared the antiproliferative effects of 1,25-dihydroxyvitamin D₃ and four synthetic analogues in two human colon cancer cell lines, HT-29 and SW620. Many analogues of 1,25-dihydroxyvitamin D₃ have been developed, with the goal of creating a compound which possesses the same antiproliferative and differentiating capabilities as the parent compound but lacks the hypercalcemic effects (16). The efficacy of vitamin D in the treatment of cancer is limited by its in vivo hypercalcemic effects. These analogues structurally differ from 1,25-dihydroxyvitamin D₃ by the introduction of double and triple bonds in the ring structure or by side chain modifications including addition of fluoro, oxo, and aromatic groups (14, 16, 17). The biological activity of these analogues is affected by their ability to diffuse into the cells, their binding affinity for VDR, and their metabolism and degradation (12). It is not clear which of these factors play the most important role.

Analogues 1,25(OH)₂-16-ene-23-yne-D₃ and 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ have been extensively studied in leukemic cell lines. Analogue 1,25(OH)₂-16-ene-23-yne-D₃, which contains a double bond between carbons 16 and 17 and a triple bond between carbons 23 and 24 (14), has been shown to inhibit the growth of HL-60, U937, and WEHI 3BD⁺ leukemic cells 4–10 times as effectively as 1,25-dihydroxyvitamin D₃ (13, 16). Similar results have been demonstrated using analogue 1,25(OH)₂-16-ene-23-yne-D₃ in Caco-2 human colon cancer cells (19). In HL-60 leukemic cells, the antiproliferative effect of analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, which in addition to the double bond at carbon 16 and the triple bond at carbon 23 has 6 fluoro groups added to carbons 26 and 27, is about 10 times that of analogue 1,25(OH)₂-16-ene-23-yne-D₃ and 40–80 times that of 1,25-dihydroxyvitamin D₃ (14). In the leukemic cells, differentiation has been assessed by an increase in the level of nonspecific esterases and the ability to reduce nitro blue tetrazolium. Both of these markers showed the differentiating capabilities of the two studied analogues to parallel their antiproliferative effects in HL-60 leukemic cells (14, 16, 20). The level of alkaline phosphatase, which is a marker of colonocyte differentiation (19), was shown to significantly increase in Caco-2 colon cancer cells after treatment of the cells with analogue 1,25(OH)₂-16-ene-23-yne-D₃, while no such increase was seen with 1,25-dihydroxyvitamin D₃. In vivo studies done in chickens showed the intestinal calcium absorption and bone calcium mobilization of analogue 1,25(OH)₂-16-ene-23-yne-D₃ to be very low, at 3.3% and 2%, respectively, of the values for 1,25-dihydroxyvitamin D₃. Analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ showed an intestinal calcium absorption of 7% and bone calcium mobilization of 10%, compared to those for 1,25-dihydroxyvitamin D₃ (14). The affinities of analogues 1,25(OH)₂-16-ene-23-yne-D₃ and 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ for the vitamin D receptor in HL-60 cells were 79% and 31%, respectively, of the value for 1,25-dihydroxyvitamin D₃.

Even though these analogues of 1,25-dihydroxyvitamin D₃ have been extensively studied in leukemic cells, limited data exist on their effects in colon cancer cells. Cross et al. (19) showed analogue 1,25(OH)₂-16-ene-23-yne-D₃ to be a relatively potent growth suppressor in Caco-2 human colon cancer cells. In this study, we chose HT-29, a moderately differentiated, primary human colon carcinoma cell line which we have previously shown to respond to the antipro-
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Fig. 3. Growth curve studies in SW620 human colon cancer cells, comparing the growth-inhibitory effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) with those of analogues 1,25(OH)₂-16-ene-23-yne-D₃, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃ and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃. Cell counts on day 6 are expressed as percentage of control. No growth inhibition was seen with any of these agents in SW620 cells.

Liferative effects of 1,25-dihydroxyvitamin D₃, and SW620, a poorly differentiated metastatic human colon carcinoma cell line which was unresponsive to the same treatment (9). Cell lines with moderate (HT-29) and very low (SW620) levels of VDR were chosen to assess the correlation between the antiproliferative effects of these synthetic analogues and the level of receptor. Each cell line was assessed by proliferation studies which use tritiated thymidine incorporation into DNA as an index of proliferative rate. Growth curve studies analyzed the increase in cell count over a 6-day period and the soft agar assay measured the anchorage-independent clonogenic growth. The results of all three techniques correlated well. The growth of HT-29 cells was most significantly inhibited by analogues 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃. These analogues reduced the proliferative rate of HT-29 cells twice as effectively as did 1,25-dihydroxyvitamin D₃ and analogue 1,25(OH)₂-16-ene-23-yne-D₃. These results are similar to those seen in leukemic cells, where the introduction of a double bond at carbon 16 and a triple bond at carbon 23 of 1,25-dihydroxyvitamin D₃ makes it a more potent inhibitor of growth and the addition of 6 fluoro groups at carbons 26 and 27 makes it an even more significant antiproliferative agent (14). The data in HT-29 cells differ from those of the leukemic cells in the degree of inhibition. This is also true of the growth suppression by analogue 1,25(OH)₂-16-ene-23-yne-D₃ in Caco-2 colon cancer cells (19). Cross et al. (19) showed the antiproliferative effect of this analogue to be 5–10-fold higher than that of 1,25-dihydroxyvitamin D₃.

In HL-60 cells, the concentration of analogues at which antiproliferative effects were seen also induced differentiation (16). Using alkaline phosphatase and CEA, both of which have been used as markers of differentiation in colon cancer cells (16, 19), we noted that the levels of these markers in HT-29 or SW620 cells did not show a significant increase upon treatment with 1,25-dihydroxyvitamin D₃ or any of the analogues at 10⁻⁸ M. Cross et al. (19) showed a significant increase in the level of alkaline phosphatase production by Caco-2 colon cancer cells upon treatment with analogue 1,25(OH)₂-16-ene-23-yne-D₃ at a concentration of 10⁻⁸ M. This difference between these two colon cancer cell lines may be attributed to the fact that Caco-2 cells undergo spontaneous differentiation when grown past the point of confluence.

In the competitive binding assays done for each analogue, the three most potent analogues showed the highest RCI values. The affinity of analogues 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₃, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃ for the VDR appears to be similar to that of 1,25-dihydroxyvitamin D₃. Ana-
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Fig. 4. [3H]Thymidine incorporation into DNA in HT-29 and SW620 colon cancer cells. After treatment with 10^{-8} M 1,25-dihydroxyvitamin D_{3} (1,25(OH)_{2}D_{3}) or analogues 1,25(OH)_{2}-16-ene-23-yne-D_{3}, 1,25(OH)_{2}-26,27-F_{6}-16-ene-23-yne-D_{3}, 1,25(OH)_{2}-16,23Z-diene-26,27-F_{6}-D_{3}, or 1,25(OH)_{2}-16,23E-diene-26,27-F_{6}-D_{3}, the proliferation rate was assessed by measuring [3H]thymidine incorporation into DNA. Thymidine uptake at different concentrations is expressed as a percentage of the control in each cell line. Inhibition of proliferation was significantly reduced (P < 0.05) in HT-29 cells by all four analogues (*). None of the agents inhibited the rate of thymidine uptake in SW620 cells.

Fig. 5. Soft agar assay showing the effects of 10^{-8} M concentrations of 1,25-dihydroxyvitamin D_{3} (1,25(OH)_{2}D_{3}) and analogues 1,25(OH)_{2}-16-ene-23-yne-D_{3}, 1,25(OH)_{2}-26,27-F_{6}-16-ene-23-yne-D_{3}, 1,25(OH)_{2}-16,23Z-diene-26,27-F_{6}-D_{3}, and 1,25(OH)_{2}-16,23Z-diene-26,27-F_{6}-D_{3} on the anchorage-independent clonogenic growth of HT-29 and SW620 colon cancer cells. No inhibition was seen in SW620 cells. HT-29 cells showed significant (P < 0.05) growth inhibition with 1,25-dihydroxyvitamin D_{3} and all four analogues (*).
Table 1  Relative competitive indices for 1,25-dihydroxyvitamin D₃ and four analogues in the HT-29 human colon cancer cell line

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<td>1,25-Dihydroxyvitamin D₃</td>
<td>100</td>
</tr>
<tr>
<td>1,25(OH)₂-16-ene-23-yne-D₃</td>
<td>59</td>
</tr>
<tr>
<td>1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃</td>
<td>95</td>
</tr>
<tr>
<td>1,25(OH)₂-16,23E-diene-26,27-F₆-D₃</td>
<td>93</td>
</tr>
<tr>
<td>1,25(OH)₂-16,23Z-diene-26,27-F₆-D₃</td>
<td>100</td>
</tr>
</tbody>
</table>

In conclusion, this study analyzes the antiproliferative and differentiating effects of four synthetic analogues of 1,25-dihydroxyvitamin D₃ in HT-29 and SW620 human colon cancer cell lines. All four analogues show very significant growth inhibition in the cell line with moderate levels of VDR and no inhibition in the cell line with low levels of VDR. In light of the potent antiproliferative effect of these minimally calcemic analogues of 1,25-dihydroxyvitamin D₃ and the correlation between the level of VDR and the degree of growth inhibition, in vivo studies using a tumor xenograft model and a colon carcinogenesis model may shed light on the efficacy of these analogues in the treatment and prevention of colon carcinoma in VDR-positive tumors.

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