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


Department of Surgery, Lombardi Cancer Research Center, Georgetown University Hospital, Washington DC 20007 (M. S., R. R. B., F. D., L. M. S., R. J. N., R. V. B., S. R. T. E.), and Hoffman-La Roche Company, Nutley, New Jersey 07110 [M. R. U.]

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 cell lines, 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-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. Growth of HT-29 cells was significantly inhibited by all four analogues at 10⁻⁴ 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 in 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, 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-ene-23-yne-26,27-hexafluoro-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 Hoffman-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, 5 mM glutamine, and 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 Instar 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-

Received 1/31/94; accepted 5/23/94.

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.

1 To whom requests for reprints should be addressed, at Department of Surgery, 4PHC, Georgetown University Hospital, 3800 Reservoir Road, N.W., Washington DC 20007.

2 The abbreviations used are: VDR, vitamin D receptor(s); CEA, carcinoembryonic antigen; RCI, relative competitive index; 1,25(OH)₂16-ene-23-yne-D₃; 1,25(OH)₂26,27-hexafluoro-16-ene-23-yne-D₃; 1,25(OH)₂26,27-F₂-16-ene-23-yne-D₃; 1,25-dihydroxy-26,27-hexafluoro-16-ene-23-yne-D₃; 1,25-dihydroxy-16,23E-diene-26,27-F₂-D₃; 1,25-dihydroxy-16,23E-diene-26,27-hexafluoro-D₃; 1,25-dihydroxy-16,23Z-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 × 10⁶ 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-D₂, or analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₂ at concentrations ranging from 10⁻¹² m 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-D₂, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₂, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₂, or 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₂ (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 × 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-D₂, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₂, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₂, or 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₂ 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 × 10⁴) were plated in 35-mm tissue culture dishes in IMEM with 5% charcoal-stripped fetal calf serum and 2 mM glutamine (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.

**DIFFERENTIATION ASSAYS.**

HT-29 and SW620 cells were plated in 162-cm² tissue culture flasks. When 80% confluency 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-D₂, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₂, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₂, or 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₂ at a concentration of 10⁻⁸ m, was added. Forty-eight h later, fresh treatment medium was added. Ninety-six h after incubation with treatment medium, i.e., control medium or medium containing 1,25-dihydroxyvitamin D₃ or analogues 1,25(OH)₂-16-ene-23-yne-D₂, 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₂, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₂, or 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₂ 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. Each point represents the mean and SD of at least three experiments. Results were compared using a linear regression using least-squares fitting. Error bars represent 1 SD of the data points fitted 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-D₂, and analogue 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₂ 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-D₂, 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-D₂, 1,25(OH)₂-16,23E-diene-26,27-F₆-D₂, and 1,25(OH)₂-16,23Z-diene-26,27-F₆-D₂ 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-
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)₂-26,27-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, which inhibited clonogenic growth by 70.1%, 74.2%, and 77.6%, 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 its three analogues did not cause any reduction in the growth of SW620 cells in soft agar.

**Differentiation Assays**

**Alkaline Phosphatase Assay.** Compared to control, treatment of HT-29 or SW620 cells with a 10⁻⁸ M concentration of 1,25-dihydroxyvitamin D₃ or analogues 1,25(OH)₂-16-ene-23-yne-D₃ did not cause a significant increase in alkaline phosphatase secretion (Fig. 6). In SW620 cells, these values actually decreased in the cells treated with the analogues. Sodium butyrate, a known differentiating agent, caused significant increases in the alkaline phosphatase production of both cell lines. Alkaline phosphatase secretion levels in both cell lines and among the control group and four treatment groups were similar, ranging from 0.8 to 1.4 units/mg of protein.

**Carcinoembryonic Antigen Assay.** Treatment of HT-29 cells with 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 did not cause an increase in the production of CEA, relative to the control cells (Fig. 6). Production was about 100 ng/mg of protein. As expected, treatment with sodium butyrate caused a significant increase in CEA production. 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 affinity of the analogues for the vitamin D receptor was 59%, 95%, 93%, and 100%, respectively (Table 1).
GROWTH SUPPRESSION BY VITAMIN D ANALOGUES

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 10⁻¹⁰ 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 into 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 leukemia 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-
Fig. 3. Growth curve studies in SW620 human colon cancer cells, comparing the growth-inhibitory effects of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) with those of 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)$_2$-16,23E-diene-26,27-F$_6$-D$_3$, and 1,25(OH)$_2$-16,23Z-diene-26,27-F$_6$-D$_3$. Cell counts on day 6 are expressed as percentage of control. No growth inhibition was seen with any of these agents in SW620 cells.

The proliferative effects of 1,25-dihydroxyvitamin D$_3$, 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 used 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-F$_6$-16-ene-23-yne-D$_3$, 1,25(OH)$_2$-16,23E-diene-26,27-F$_6$-D$_3$, and 1,25(OH)$_2$-16,23Z-diene-26,27-F$_6$-D$_3$. These analogues reduced the proliferative rate of HT-29 cells twice as effectively as did 1,25-dihydroxyvitamin D$_3$ and analogue 1,25(OH)$_2$-16-ene-23-yne-D$_3$. 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 D$_3$ 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-D$_3$ 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 D$_3$. SW620 human colon cancer cells did not undergo any growth inhibition with 1,25-dihydroxyvitamin D$_3$ or the four synthetic analogues. In studies of leukemic cells, treatment of WEHI 3BD$^-$ and KG-1 cells with 1,25-dihydroxyvitamin D$_3$ failed to reduce their proliferative rate or to induce differentiation (14, 20). These cells, which like SW620 cells are immature and undifferentiated, did not respond to treatment with analogue 1,25(OH)$_2$-16-ene-23-yne-D$_3$, either. In our earlier study, we have noted that, among colon cancer cell lines, a correlation exists between the level of VDR and the level of antiproliferative effect of 1,25-dihydroxyvitamin D$_3$ (9). SW620 is an undifferentiated cell line with low levels of VDR which did not respond to treatment with 1,25-dihydroxyvitamin D$_3$. Like the two immature and undifferentiated leukemic cell lines, SW620 did not respond to treatment with the four synthetic analogues.

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 D$_3$ 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-D$_3$ 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-F$_6$-16-ene-23-yne-D$_3$, 1,25(OH)$_2$-16,23E-diene-26,27-F$_6$-D$_3$, and 1,25(OH)$_2$-16,23Z-diene-26,27-F$_6$-D$_3$ for the VDR appears to be similar to that of 1,25-dihydroxyvitamin D$_3$. Ana-
GROWTH SUPPRESSION BY VITAMIN D ANALOGUES

Fig. 4. [$^3$H]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)$_2$-16,23E-diene-26,27-F$_6$-D$_3$, or 1,25(OH)$_2$-16,23Z-diene-26,27-F$_6$-D$_3$, the proliferation rate was assessed by measuring [$^3$H]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)$_2$-16,23E-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

<table>
<thead>
<tr>
<th>Name</th>
<th>RCI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<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>

Fig. 6. Assessment of the differentiating effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and analogues 1,25(OH)₂-16-ene-23-yne-D₃ and 1,25(OH)₂-26,27-F₆-16-ene-23-yne-D₃ on HT-29 and SW620 colon carcinoma cells, by assaying for changes in alkaline phosphatase or CEA activity. None of the agents caused any significant change in the levels of these markers in either cell line.

Fig. 7. Competitive binding assay comparing the affinities 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 for VDR to that of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in HT-29 cells. This graph shows the reciprocal of the percentage of maximal binding as a function of the ratio of the concentration of each competitor to that of [³H]-1,25-dihydroxyvitamin D₃. RCI values were determined by dividing the slopes of the lines for the analogues (Comp) by that for 1,25-dihydroxyvitamin D₃, which was assigned an RCI of 100%. * Statistically significant difference in RCI, compared to that of 1,25-dihydroxyvitamin D₃.

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.

ACKNOWLEDGMENTS

The authors would like to express appreciation to June E. Bishop (Department of Biochemistry, University of California, Riverside, CA) and Robert Clarke (Lombardi Cancer Center, Georgetown University, Washington, DC) for their assistance with the competitive binding assay.

REFERENCES

4. DeLaca, H. F., and Ostrem, V. The relationship between the vitamin D system and


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