Vitamin D₂ Analog 19-nor-1,25-Dihydroxyvitamin D₂: Antitumor Activity Against Leukemia, Myeloma, and Colon Cancer Cells

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Background: 1,25-Dihydroxyvitamin D₃ inhibits growth of several types of human cancer cells in vitro, but its therapeutic use is hampered because it causes hypercalcemia. 19-nor-1,25-Dihydroxyvitamin D₂ (paricalcitol) is a noncalcemic vitamin D analog that is approved by the Food and Drug Administration for the treatment of secondary hyperparathyroidism. We investigated the antitumor activity and mechanism of action of paricalcitol in vitro and in vivo. Methods: Effects of paricalcitol on proliferation, the cell cycle, differentiation, and apoptosis were examined in cancer cell lines. Effects on tumor growth were examined with colon cancer cell xenografts in nude mice (five in the experimental group and five in the control group). The interaction of paricalcitol with the vitamin D receptor (VDR) in mononuclear spleen cells and myeloid stem cells from wild-type and VDR knockout mice was examined. All statistical tests were two-sided. Results: Paricalcitol inhibited the proliferation of myeloid leukemia cell lines HL-60, NB-4, and THP-1 cells at an effective dose that inhibited growth 50% (ED₅₀) of 2.4–5.8 × 10⁻⁹ M by inducing cell cycle arrest and differentiation. Paricalcitol inhibited the proliferation of NCI-H929 myeloma cells at an ED₅₀ of 2.0 × 10⁻¹⁰ M by inducing cell cycle arrest and apoptosis. Paricalcitol also inhibited the proliferation of colon cancer cell lines HT-29 (ED₅₀ = 1.7 × 10⁻⁹ M) and SW837 (ED₅₀ = 3.2 × 10⁻⁸ M). HT-29 colon cancer xenografts in paricalcitol-treated nude mice were smaller (1044 mm³ and 1752 mm³, difference = 708 mm³, 95% confidence interval = 311 to 1104 mm³; P = .03) and weighed less (1487 mg and 4162 mg, difference = 2675 mg, 95% confidence interval = 2103 to 3248 mg; P<.001) than those in vehicle-treated mice. Paricalcitol induced committed myeloid hematopoietic stem cells from wild-type but not from VDR knockout mice to differentiate as macrophages. Conclusion: Paricalcitol has anticancer activity against myeloid leukemia, myeloma, and colon cancer cells that may be mediated through the VDR. Because it has been approved by the Food and Drug Administration, clinical trials of this agent in certain cancers are reasonable. [J Natl Cancer Inst 2003;95:896–905]
lines (HL-60, NB-4, THP-1, and U937), lymphoma cell lines (Raji, Ramos, Daudi, Jurkat, Jeko-1, and JUDHL), and myeloma cell lines (RPMI-8226, ARH-77, and NCI-H929) were grown in RPMI-1640 medium with 10% fetal calf serum (FCS). Breast cancer cell lines (MCF-7 and MDA-MB-231), brain cancer cell lines (U343, U118, U138, U373, and U87), and colon cancer cell lines (HT-29, SW837, SW480, SW620, and HCT116) were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% FCS. The endometrial carcinoma cell line AN-3 was maintained in the α modification of minimal essential medium (α-MEM; Gibco BRL, Grand Island, NY) with 10% FCS. Paricalcitol and 1,25(OH)2D3 were obtained from Abbott Laboratories (Abbott Park, IL).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays for Cell Proliferation and Viability

To measure cell proliferation and viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) at 5 mg/mL. Approximately 1 × 10^4 cells per well were incubated in culture medium for 96 hours in 96-well plates, and then 10 μL of the MTT solution was added. After a 4-hour incubation, 100 μL of solubilization solution (20% sodium dodecyl sulfate [SDS]) was added, and the mixture was incubated at 37 °C for 16 hours. In this assay, MTT is cleaved to an orange formazan dye by metabolically active cells. The absorbance of the formazan product is measured with an enzyme-linked immunosorbent assay reader at 540 nm.

Soft Agar Colony Assay

The two-layer soft agar system (10) was used. Cells were removed from culture plates with trypsin and washed. Cells in single-cell suspension were counted. Approximately 1 × 10^5 cells in 400 μL of medium per well were plated into 24-well flat-bottom plates and incubated for 14 days at 37 °C in a humidified atmosphere containing 5% CO2/95% air, and then the colonies were counted.

Cell Cycle Analysis

Cultured cells were treated with 1 × 10^-7 M 1,25(OH)2D3, 1 × 10^-7 M paricalcitol, or a vehicle control (10% FCS in RPMI-1640 medium containing 0.01% ethanol) for 3 or 4 days. All cells (those in suspension and those attached to the culture dish) were collected, washed, suspended in ice-cold PBS, fixed in 75% chilled methanol at 4 °C, and stained with propidium iodine (PI). Cell cycle status was analyzed on a Becton Dickinson Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).

Western Blot Analysis

Cells were washed twice in PBS, suspended in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, phenylmethylsulfonyl fluoride at 100 μg/mL, aprotonin at 2 μg/mL, pepstatin at 1 μg/mL, and leupeptin at 10 μg/mL), and placed on ice for 30 minutes. After centrifugation at 15,000g for 15 minutes at 4 °C, the suspension was collected. Protein concentrations were quantitated by using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s recommendation. Proteins in whole lysates (40 μg) were resolved by SDS–polyacrylamide gel electrophoresis in a 4%–15% gel, transferred to a polyvinylidene difluoride membrane (Immobilon; Amersham, Arlington Heights, IL), and probed sequentially with antibodies against the following proteins: p21<sup>WAF1</sup>, p27<sup>KIP1</sup>, PTEN, BCL2, BAX, C-MYC, cyclin D1, cyclooxygenase (COX-1), COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed with the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL).

Measurement of Apoptosis

The cells undergoing apoptosis were detected by morphologic changes including nuclear shrinkage and cellular fragmentation by staining cells with Hoechst 33258 (Bisbenzimide H 33258; Sigma). Cells in the sub-G1 population, an indication of the number of apoptotic cells present, were detected by cell cycle analysis with flow cytometry. The terminal deoxynucleotidyl transferase-mediated uridine 5’-triphosphate nick end labeling (TUNEL) assay was also used to detect and quantify apoptosis (In Situ Cell Death Detection kit; Roche, Indianapolis, IN).

Measurement of Cell Surface CD14 Antigen on HL-60 Cells

HL-60 myelocytic leukemia cells were treated with 1 × 10^-7 M 1,25(OH)2D3, 1 × 10^-7 M paricalcitol, or a vehicle control (10% FCS in RPMI-1640 medium containing 0.01% ethanol) for 4 days and examined for CD14 expression with CD14 antibody (DAKO, Carpenteria, CA) by flow cytometry, as described previously (12). Murine immunoglobulin G1 antibody (DAKO) was used as a control antibody.

Murmur Studies

BNX nu/nu mice were purchased from Harlan (Indianapolis, IN) at 8 weeks of age and cared for in accordance with the guidelines of Cedars-Sinai Research Institute. They were maintained in pathogen-free conditions and fed irradiated chow. A total of 1 × 10^6 HT-29 cells in 0.1 mL of Matrigel (Collaborative Biological Products, Bedford, MA) was injected subcutaneously into bilateral flanks of each mouse, resulting in the formation of two tumors per mouse. The mice were assigned blindly and randomly to the experimental group or the control group. Treatment started the day after HT-29 cells were injected and continued for 4 weeks. The five control mice received vehicle (100 μL of PBS containing 0.024% ethanol per day, intraperitoneally, 3 days per week on Monday, Wednesday, and Friday) only, and the five experimental mice received paricalcitol (100 ng in 100 μL of PBS containing 0.024% ethanol per day, intraperitoneally, 3 days per week on Monday, Wednesday, and Friday). Tumor sizes were measured every week and calculated by the formula A × B × C × 0.5236, where A is the length, B is the width, and C is the height, all measured in millimeters. Tumor size and weight from each mouse were calculated as the total of the two bilateral tumors per mouse. After 4 weeks, blood was collected to measure the level of serum calcium. All mice were killed at the end of the study, and tumors were fixed in 10% neutral buffered formalin and embedded in paraffin for histologic analysis.

Measurement of Serum Calcium in Mice

To measure the serum calcium levels in mice, we used Sigma Diagnostics calcium reagent (Sigma) containing o-cresolphosphate.
thalein, which interacts with calcium to form a purple-colored complex. The colored complex was directly quantified with an enzyme-linked immunosorbent assay reader at 575 nm and absorbance (A) of the blank, standard, and sample were measured. Calcium concentration of the sample (mg/dL) was calculated as 

\[ A_{\text{sample}} - A_{\text{blank}} = A_{\text{standard}} - A_{\text{blank}} \times 10. \]

**Vitamin D Receptor Knockout Mice and Soft Gel Colony-Forming Assay**

Five vitamin D receptor (VDR) knockout mice were generated, and genotypes were determined by Southern blot analysis as described previously (16). Five wild-type littermates were used as controls. Mice were killed by cervical dislocation. Bone marrow was flushed from isolated femurs with α-MEM containing 10% FCS by the use of a 26-gauge needle and syringe. Isolated spleens were injected with DMEM (Gibco BRL) containing 10% FCS and crushed with forceps to release cells. Mononuclear cells from bone marrow or spleen were separated by Ficoll–Hypaque density centrifugation (Amersham Pharmacia, Uppsala, Sweden).

For the soft gel assay, resuspended mononuclear bone marrow cells (2 \( \times \) 10^4 cells per mL) and growth factors to final concentrations of 1% methylcellulose, 30% FCS, granulocyte–macrophage colony-stimulating factor (10 ng/mL), interleukin 3 (10 ng/mL), 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, and 2 mM L-glutamine, as described previously, were added to methylcellulose medium M3234 (StemCell Technologies, Vancouver, British Columbia, Canada) (16). Cells were plated in six-well plates in 1 mL of medium and incubated at 37°C in a humidified atmosphere containing 5% CO2/95% air. Colonies were counted after 2 weeks. Colony type was established by morphology. To ensure accurate determination, representative colonies were plucked from the plates, centrifuged onto slides, stained with Wright–Giemsa stain, and examined by light microscopy. The number of granulocyte colonies, macrophage colonies, and mixed granulocyte/macrophage colonies was counted.

**Polymerase Chain Reaction Analysis**

RNA extraction and reverse transcription were done with TRIzol (Invitrogen, Carlsbad, CA) and reverse transcriptase (Promega, Madison, WI); 20 µL of cDNA was prepared from 1 µg of RNA. cDNAs were amplified by polymerase chain reaction (PCR) with specific primers for 24-hydroxylase and 18S rRNA, with 25 PCR cycles for 18S rRNA and 32 cycles for 24-hydroxylase. The PCR primers used to amplify 24-hydroxylase were 5'-GCTTACGCCGAGTGTACCAT-3' (forward) and 5'-ATGAGCACTGTTCCTTTGGG-3' (reverse), and those used to amplify 18S rRNA were 5'-AAACGGCTACCACTCCAAAG-3' (forward) and 5'-CCTCCAAATGATCCCTC GTTA-3' (reverse). Annealing temperatures for the PCRs were 56°C for 24-hydroxylase and 58°C for 18S rRNA. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed.

**Statistical Analysis**

For in vivo studies in mice, the differences of tumor sizes and weights between mice in the control group and mice in the experimental group at the end of the study were analyzed by Student’s t test. These data met the assumptions for Student’s t test. All statistical tests were two-sided.

**RESULTS**

**Paricalcitol and Clonal Proliferation of Leukemia and Colon Cancer Cell Lines In Vitro**

We examined whether paricalcitol had antitumor activity in human cancer cell lines derived from breast, brain, colon, and uterine cancers, as well as from myeloid leukemia, lymphoma, and myeloma. We first used the rapid MTT assay with a relatively short 4-day exposure to paricalcitol (data not shown) to show that paricalcitol had antiproliferative activity in myeloid leukemia cells (HL-60, NB-4, and THP-1), myeloma cells (NCI-H929), and colon cancer cells (HT-29 and SW837). We then tested whether paricalcitol affected the ability of the same cell lines to form colonies in soft agar (Fig. 1). The concentrations of paricalcitol that caused 50% inhibition (ED_{50}) of clonal growth in soft agar were as follows: for HL-60 cells, 2.4 \( \times \) 10^{-9} M; for NB-4 cells, 3.4 \( \times \) 10^{-9} M; for THP-1 cells, 5.8 \( \times \) 10^{-9} M; for HT-29 cells, 1.7 \( \times \) 10^{-8} M; for SW837 cells, 3.2 \( \times \) 10^{-8} M; and for NCI-H929 cells, 2.0 \( \times \) 10^{-10} M. These ED_{50} values were comparable to those obtained with the physiologically active form of vitamin D$_3$ 1,25(OH)$_2$D$_3$ (Fig. 1).

**Paricalcitol and Myeloid Leukemia Cells In Vitro**

For cell cycle analysis, HL-60 cells were treated with 1 \( \times \) 10^{-7} M paricalcitol or vehicle for 72 hours and examined by flow cytometry. Paricalcitol-treated HL-60 cells accumulated in the G$_0$/G$_1$-phase populations (64% [mean], a 16% [95% confidence interval [CI] = 11% to 21%] increase compared with vehicle-treated cells [48%]) and in the G$_2$/M-phase populations (27%, a 17% [95% CI = 6% to 28%] increase compared with vehicle-treated cells [10%]). A concomitant decrease was observed in the proportion of cells in the S-phase population (9%, a 33% [95% CI = 22% to 44%] decrease compared with vehicle-treated cells [42%]) (Fig. 2, A).

To explore the mechanism of action of paricalcitol, we examined the expression of cyclin-dependent kinase inhibitors (CDKIs) p21^{WAF1} and p27^{KIP1}. Increased expression of p21^{WAF1} and p27^{KIP1} was observed in paricalcitol-treated cells and in 1,25(OH)$_2$D$_3$-treated cells but not in vehicle-treated cells. Both paricalcitol and 1,25(OH)$_2$D$_3$ (each at 1 \( \times \) 10^{-7} M for 72 hours) increased the expression of p21^{WAF1} by approximately sevenfold and p27^{KIP1} by approximately sixfold in the HL-60 cells (Fig. 2, B). Thus, paricalcitol increased the expression of CDKIs, and this result may reflect the cell cycle arrest induced by paricalcitol.

Several analogs of vitamin D have been shown to increase the expression of the potential tumor suppressor gene PTEN in a leukemic cell line, indicating that this protein may help to mediate the antitumor activity of vitamin D and its analogs (12). PTEN is a phosphatase that targets activated phosphatidylinositol 3-kinase (PI3K). We measured the levels of PTEN by western blot analysis. After treatment with 1 \( \times \) 10^{-7} M paricalcitol for 72 hours, PTEN expression increased sevenfold in HL-60 cells and 25-fold in NB-4 myeloid leukemia cells, compared with the increase in levels in vehicle-treated cells, essentially as observed when these cells were treated with 1 \( \times \) 10^{-7} M 1,25(OH)$_2$D$_3$ for 72 hours (Fig. 2, B).

Because vitamin D and its analogs induce monocytic differentiation of some myeloid leukemia cells as a result of their antiproliferative activity (1,3,5), we examined the potency of paricalcitol to stimulate monocytic differentiation of HL-60
cells. Paricalcitol induced monocyte/macrophage-like differentiation of HL-60 cells, as measured by the increased expression of cell surface marker CD14. Paricalcitol (1 × 10^{-7} M for 96 hours) induced 65% of HL-60 cells to express CD14, and 1,25(OH)_{2}D_{3} (1 × 10^{-7} M for 96 hours) induced 54% of HL-60 cells to express CD14 (Fig. 2, C). Morphologic examination clearly showed monocytic differentiation of HL-60 cells treated with 1 × 10^{-7} M paricalcitol for 120 hours. Untreated control HL-60 cells are large with round or oval nuclei, prominent nucleoli, and amphophilic cytoplasm, whereas paricalcitol-treated cells showed monocytoid differentiation with oval, irregular, or indented nuclei and abundant vacuolated cytoplasm (Fig. 2, D).

**Paricalcitol and Human Myeloma Cells In Vitro**

Dose-dependent antiproliferative activity of paricalcitol was higher in NCI-H929 myeloma cells than in RPMI-8226 and ARH-77 myeloma cells (data not shown), and so the effect of paricalcitol on the cell cycle of NCI-H929 myeloma cells was examined by flow cytometry. NCI-H929 cells were treated with 1 × 10^{-7} M paricalcitol, 1 × 10^{-7} M 1,25(OH)_{2}D_{3}, or vehicle for 72 hours, and cell cycle analysis was performed. In paricalcitol-treated cultures, 58% of the cells were in the G1/G0-phase population and 21% of the cells were in the sub-G1 population. In 1,25(OH)_{2}D_{3}-treated cultures, 62% of the cells were in the G1/G0-phase population and 16% of cells were in the sub-G1 population. In vehicle-treated cultures, 52% of the cells were in the G1/G0-phase population and 4% of the cells were in the sub-G1 population (Fig. 3, A).

The morphologic examination showed increased changes typical of apoptosis, including nuclear shrinkage and cellular fragmentation, of the NCI-H929 cells treated with 1 × 10^{-7} M paricalcitol or 1 × 10^{-7} M 1,25(OH)_{2}D_{3} for 96 hours compared with the morphology of control cells (data not shown). As shown in Fig. 3, A, these treatments also caused these cells to accumulate in the sub-G1 population, which is typical of cells undergoing apoptosis. Thus, the antiproliferative effect of paricalcitol in NCI-H929 cells may be associated with induction of apoptosis. Apoptosis was next examined with the TUNEL assay. NCI-H929 cells were treated with 1 × 10^{-7} M paricalcitol, 1 × 10^{-7} M 1,25(OH)_{2}D_{3}, or vehicle for 96 hours and subjected to the TUNEL assay (Fig. 3, B). Thirty-one percent (95% CI = 23% to 39%) of cells in paricalcitol-treated cultures and 20% (95% CI = 16% to 24%) of cells in 1,25(OH)_{2}D_{3}-treated cultures were apoptotic. The percentage of apoptotic cells in both cultures was statistically significantly higher than that of vehicle-treated control cultures (7%, 95% CI = 5% to 9%; P = .003 and P = .001, respectively). The differences between paricalcitol-treated cultures and control cultures and between 1,25(OH)_{2}D_{3}-treated cultures and control cultures were 24% (95% CI = 16% to 30%) and 13% (95% CI = 10% to 16%), respectively. Under the same conditions, the expression of p27^{kip1} increased 3.4-fold or 2.8-fold, respectively, in paricalcitol-treated or 1,25(OH)_{2}D_{3}-treated cells (Fig. 3, C), but the expression of p21^{waf1} was essentially unaffected (data not shown). When re-treated with paricalcitol or 1,25(OH)_{2}D_{3} (each at 10^{-7} M for 72 hours), NCI-H929 cells expressed approximately 40% less antiapoptotic BCL-2 protein but expressed approximately the same amount of the proapoptotic protein BAX (Fig. 3, C).

**Paricalcitol and Colon Cancer Cells In Vitro and In Vivo**

We next investigated the antiproliferative activity of paricalcitol on various colon cancer cell lines. Cells were incubated with 1 × 10^{-7} M paricalcitol or vehicle for 96 hours, and proliferation was assessed with the MTT assay. When expressed as a percentage of the vehicle control, proliferation of HT-29 cells (50%, 95% CI = 37% to 63%) and SW837 cells (70%, 95% CI = 64% to 76%) was more sensitive to paricalcitol than that of...
SW480 cells (85%, 95% CI = 77% to 93%), and HCT116 cells were resistant to paricalcitol (95%, 95% CI = 81% to 109%) (Fig. 4, A). The mechanism of this antiproliferative effect was further studied in HT-29 cells, the colon cancer cell line most sensitive to paricalcitol. HT-29 cells were incubated with $1 \times 10^{-7} M$ paricalcitol, $1 \times 10^{-7} M 1,25(OH)_{2}D_{3}$, or the vehicle.
eraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression levels presented in the text were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Levels of p21 WAF1 and p27 KIP1 protein in paricalcitol-treated cells were measured. Levels of p21 WAF1 and p27 KIP1 proteins were measured. Levels of p21 WAF1 and p27 KIP1 proteins were measured. The expression of COX-1, relative to control cells, did not change in cells subjected to either treatment.

We then evaluated the effect of paricalcitol on HT-29 human colon cancer xenografts growing in nude mice. Beginning the day after cells were injected into the mice, paricalcitol was injected intraperitoneally 3 times per week on Monday, Wednesday, and Friday. Tumor volumes were measured weekly, and the volumes from both bilateral tumors were added to give the total volume for the mouse. Tumors were dissected and weighed. Those in paricalcitol-treated mice compared with those in vehicle-treated mice were statistically significantly smaller (1044 mm³ and 1752 mm³, respectively; difference = 708 mm³, 95% CI = 311 to 1104 mm³; \( P = .03 \)) (Fig. 5, A) and lighter (1487 mg and 4162 mg, respectively; difference = 2675 mg, 95% CI = 2103 to 3248 mg; \( P < .001 \)) (Fig. 5, B). Serum calcium levels were 9.5 mg/dL and 10.2 mg/dL in paricalcitol-treated mice and control mice, respectively; \( P = .17 \), both in the normal range.
Paricalcitol and the VDR

We examined the association between the expression of VDR and the antiproliferative effect of paricalcitol. All colon cancer cell lines examined—HT-29, SW837, SW480, and HCT116—expressed VDR protein (Fig. 6, A). No association was observed between the level of VDR protein in the various cell lines and their sensitivity to paricalcitol, as determined by the MTT assay (Fig. 4, A).

The enzyme 25-hydroxyvitamin D₃-24-hydroxylase catalyzes the first step in the catabolism of 1,25(OH)₂D₃ (19). Expression of 24-hydroxylase is transcriptionally regulated and is activated by the binding of its ligand, 1,25(OH)₂D₃, or its analog to the VDR. The VDR–ligand complex then binds to the vitamin D response element in the 24-hydroxylase promoter and activates its transcription (20,21). When HT-29 cells were incubated with 1 × 10⁻⁷ M paricalcitol, expression of 24-hydroxylase mRNA was induced within 6 hours and continued to increase for at least 24 hours (Fig. 6, B).

We next investigated whether paricalcitol activity required an intact VDR by using isolated mononuclear cells from the spleen of a wild-type mouse or a VDR knockout mouse and culturing these cells with 1 × 10⁻⁸ M paricalcitol. Within 12 hours, 24-hydroxylase mRNA was expressed in paricalcitol-treated mononuclear cells from the wild-type mouse but not in paricalcitol-treated cells from the VDR knockout mice (Fig. 6, C).

We previously observed (16) that 1,25(OH)₂D₃ stimulated myeloid stem cells to differentiate via the macrophage pathway. To determine whether paricalcitol stimulated bone marrow cells to differentiate via the same pathway, we added 1 × 10⁻⁸ M paricalcitol to soft gel cultures of murine bone marrow cells from VDR knockout mice or wild-type mice and scored colonies 2 weeks later as macrophage, granulocyte, or mixed granulocyte/macrophage colonies. The total number of colonies from wild-type murine bone marrow cells averaged 87 in untreated control cultures and 66 in paricalcitol-treated cultures; the total number of colonies from murine bone marrow cells isolated from VDR knockout mice averaged 110 in untreated control cultures and 122 in paricalcitol-treated cultures. Thus, monocytic differentiation of committed myeloid stem cells appears to require the VDR (Fig. 6, D). Myeloid stem cells from wild-type mice were tested in the soft gel assay to determine the distribution of differentiated colonies. Paricalcitol treatment increased the percentage of macrophage colonies from 32% (95% CI = 22% to 43%) in vehicle-treated control cultures to 69% (95% CI = 63% to 76%) in paricalcitol-treated cultures, decreased the percentage of mixed colonies from 43% (95% CI = 25% to 62%) in control cultures to 25% (95% CI = 13% to 38%) in paricalcitol-treated cultures, and decreased the percentage of granulocyte colonies from 23% (95% CI = 14% to 33%) in control cultures to 6% (95% CI = 3% to 15%) in paricalcitol-treated cultures. When myeloid stem cells from the knockout mice were tested in the soft gel assay, paricalcitol did not alter the percentage of macrophage colonies in control cultures (30%, 95% CI = 8% to 52%) and in paricalcitol-treated cultures (25%, 95% CI = 13% to 38%), the percentage of mixed granulocyte/macrophage colonies in control cultures (56%, 95% CI = 29% to 84%) and in paricalcitol-treated cultures (61%, 95% CI = 41% to 81%), or the percentage of granulocyte colonies in control cultures (13%, 95% CI = 6% to 21%) and in paricalcitol-treated cultures (13%, 95% CI = 4% to 23%).

DISCUSSION

We observed that the vitamin D analog paricalcitol inhibited proliferation of myeloid leukemia, myeloma, and colon cancer cell lines in vitro by modulating cell cycle progression, differentiation, and apoptosis. Paricalcitol also inhibited the in vivo growth of HT-29 human colon cancer xenografts in nude mice.
The antiproliferative activity of paricalcitol was accompanied by cell cycle arrest and changes in the expression of p21WAF1 and p27Kip1. Previous studies (22–24) showed that vitamin D analogs induce cell cycle arrest in G1/G0 phases; the arrest may also be mediated by p21WAF1 and p27Kip1. Paricalcitol arrested in HL-60 leukemia cells in G1/G0 phases and G2/M phases, arrested NCI-H929 myeloma cells in G1/G0 phases, and induced the expression of p21WAF1 and p27Kip1 in leukemia, myeloma, and colon cancer cell lines. Thus, the CDKIs may play a role in the antiproliferative effects of paricalcitol and other vitamin D analogs by reducing the ability of the tumor cells to enter S phase (25,26). The block at the G2/M-phase checkpoint has been previously reported in HL-60 cells treated with 1,25(OH)2D3 (24). The vitamin D3-mediated retardation of cells at the G2/M-phase checkpoint that is accompanied with decreased levels of p34(cdc) has been reported (27); we have not yet examined the expression of p34(cdc) in paricalcitol-treated cells.

Paricalcitol induced the expression of several tumor suppressor genes including PTEN and E-cadherin. The PTEN phosphatase can block PI3K/Akt signaling pathways, which contribute to both cell death and the inhibition of cell proliferation (28,29). PTEN mutations have been found in many human cancers (30–36). In mice, homozygous germline deletion of PTEN resulted in early embryonic lethality, but heterozygous germline deletion of PTEN was associated with an increased incidence of malignant neoplasms. Thus, PTEN appears to behave like a tumor suppressor gene by depressing the pro-growth signals of the PI3K/Akt pathway (37,38). We previously noted that 1,25(OH)2D3 and one of its analogs, 21-[3-methyl-3-hydroxy-butyl]-19-nor vitamin D3, increased the expression of PTEN in HL-60 cells (12). In this study, we showed that paricalcitol and 1,25(OH)2D3 induced PTEN expression in HL-60 and NB-4 myeloid leukemia cells.

It remains to be determined whether paricalcitol or other vitamin D compounds can inhibit growth of cells with PTEN deletions. We suspect that these compounds may do so. Previously, an intragenic deletion including MMAC1/PTEN exons 2–5 in the myeloblastic leukemia cell line HL-60 and a four-nucleotide insertion in exon 5 in the acute monocytic leukemia cell line U937 were identified (39). Vitamin D compounds can induce these cells to undergo terminal differentiation. Furthermore, PTEN mutations are present in prostate cancer cell lines (30), and 1,25(OH)2D3 can inhibit the growth and induce the differentiation of prostate cancer cells (40–42). Low levels of PTEN are detected in cells with methylated PTEN promoter regions. Because low levels of PTEN are expressed by some tumor cells, including endometrial, breast, colon, and prostate cancer cell lines (30,43,44), and several vitamin D analogs that slow the growth of these cells, it should be determined whether the antiproliferative activity of paricalcitol in these cells is associated with the concomitant demethylation of the PTEN promoter and the increased expression of PTEN.

Paricalcitol-treated colon cancer cells had increased expression of E-cadherin, a transmembrane linker protein located in intercellular adherent junctions, which maintain the adhesive and polarized phenotype of epithelial cells (45,46). Loss of E-cadherin expression occurs as cells acquire the capacity to invade during the transition from adenoma to carcinoma (47,48). E-cadherin is a tumor suppressor gene, and its loss is associated with poor prognosis (49–51). E-cadherin also regulates β-catenin by holding it in place at the cell membrane. Loss of E-cadherin allows β-catenin to interact with cytoplasmic adenomatous polyposis coli (APC), which helps to mediate the ubiquitination and degradation of β-catenin. Mutation of the APC gene, which occurs frequently in the development of colon cancer, can result in β-catenin accumulating in the nucleus and acting as a co-stimulatory protein for the T-cell factor (TCF) family of transcription factors. Activation of these transcriptional factors increases the expression of a number of pro-growth genes, including cyclin D1 and c-myc (52–56).

A recent study (57) suggested that ligand-activated VDR competes with TCF-4 for binding to β-catenin in the nucleus; this competition releases β-catenin so that it can translocate from the nucleus to the E-cadherin complex at the plasma membrane and thus inhibit the transcriptional regulatory activity of TCF. We found that paricalcitol-treated HT-29 cells had increased levels of E-cadherin and decreased levels of cyclin D1 and MYC, the latter two being subject to TCF/β-catenin activation in such cells. Our results are consistent with the anticancer effects of paricalcitol being associated with its modulation of the E-cadherin/β-catenin/TCF pathway. Future studies should examine the levels and cellular location of β-catenin in these paricalcitol-treated cells.

Paricalcitol-induced apoptosis in NCI-H929 myeloma cells was accompanied by the decreased expression of BCL-2 protein without an alteration in the level of BAX protein. In another study (58), treatment with the vitamin D3 analog EB1089 inhibited the proliferation of NCI-H929 cells, decreased the expression of BCL-2, increased caspase 3 activity and p38 kinase activity, and suppressed p44 extracellular signal-related kinase activity during apoptosis. The effect of paricalcitol on p38 and extracellular signal-related kinase was not investigated.

Epidemiologic studies suggest that the use of nonsteroidal anti-inflammatory drugs decreases the risk of developing certain malignancies, including colon cancer (59,60). The major targets of these drugs are COX-1 and COX-2, which participate in the conversion of arachidonic acid to prostaglandins (61). COX-1 is expressed more or less ubiquitously in the body and has many physiologic activities, including maintenance of the gastrointestinal mucosa and various renal and platelet functions (62,63). In contrast, COX-2 is induced by various inflammatory stimuli, including cytokines, growth factors, and carcinogens, and may promote the growth of cancerous and precancerous cells (64). COX-2 expression is elevated in various malignancies (17,18) and is, therefore, a reasonable target for cancer chemoprevention. Selective COX-2 inhibitors suppress carcinogenesis in rodent models, germline disruption of the COX-2 gene inhibits polyp formation in mice with a genetic predisposition to develop these tumors (65), and a selective COX-2 inhibitor reduces the polyp burden in patients with familial adenomatous polyposis (66). Furthermore, the number of polyps decreased after 1,25(OH)2D3 or its analog was given to mice with the same germline alteration as found in the patients with familial adenomatous polyposis (67,68).

These reports prompted us to investigate the effect of paricalcitol on the expression of COX-2 in colon cancer cells. In HT-29 and SW483 colon cancer cells, treatment with paricalcitol or 1,25(OH)2D3 decreased expression of COX-2, compared with vehicle-treated control cells, but not of COX-1, suggesting that these vitamin D analogs acted as selective COX-2 inhibitors. The mechanism by which vitamin D inhibits COX-2 expression in colon cancer cells remains to be elucidated.
We also examined whether paricalcitol mediated its effects through the VDR. We found no association between the level of VDR expression in a colon cancer cell line and its sensitivity to paricalcitol (Fig. 6, A). This result was not surprising because we (69) and others (70) have previously found little association between overall cellular levels of VDR and responsiveness to vitamin D₃ analogs, and the activity of some vitamin D₃ compounds may be mediated independently of the VDR through a nongenomic pathway. We did, however, show that the expression of 25-hydroxyvitamin D₃-24-hydroxylase, a target gene of activated VDR, was induced in paricalcitol-treated cells. We also showed that paricalcitol required the VDR to mediate macrophage differentiation of myeloid hematopoietic stem cells. 25-Hydroxyvitamin D₃-24-hydroxylase was not induced by paricalcitol in the cells from VDR knockout mice but was induced by paricalcitol in cells from wild-type mice. Thus, the VDR appears to be necessary, but not sufficient, to ensure that cancer cells will be sensitive to paricalcitol.

In this study, we showed that paricalcitol and 1,25(OH)₂D₃ at similar concentrations had similar biologic activities in vitro. The serum half-life of both analogs is also similar (http://www.fda.gov/cder/foi/). However, because paricalcitol is much less likely to cause hypercalcemia in patients, physicians can administer higher doses, resulting in higher serum concentrations. Thus, a typical dose of 1,25(OH)₂D₃ is 0.5–1.0 µg every other day, for a peak serum level of 40–60 pg/mL, whereas a typical dose of paricalcitol is 2.8–7.5 µg every other day (and up to 16.8 µg has been safely given), for a peak serum level of 1850 pg/mL. Therefore, paricalcitol can be given at higher doses than 1,25(OH)₂D₃ to obtain higher serum levels without toxicity and to result in greater efficiency in the treatment of vitamin D₃-sensitive cancers.

In conclusion, paricalcitol has potent anticancer activity against myeloid leukemia and myeloma cells in vitro and colon cancer cells in vitro and in vivo through modulation of the cell cycle, differentiation, and apoptosis. Because paricalcitol has been approved by the Food and Drug Administration for the treatment of secondary hyperparathyroidism, innovative clinical trials of this agent for preleukemia (myelodysplastic syndrome), maintenance therapy for acute myeloid leukemia and multiple myeloma, and adjuvant therapy for colon cancer are reasonable to consider.

**References**


(26) Yang ZY, Perkins ND, Ohno T, Nabel EG, Nabel GJ. The p21 cyclin-


