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Vitamin D_3 Analogs and Their 24-Oxo Metabolites Equally Inhibit Clonal Proliferation of a Variety of Cancer Cells but Have Differing Molecular Effects

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Abstract The seco-steroid hormone, 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) binds to a specific nuclear receptor that acts as a ligand-inducible transcription factor. The resulting genomic effects include partial arrest in G_0/G_1 of the cell cycle and induction of differentiation; these effects have been observed in various types of cancer. Recently, we produced enzymatically the natural 24-oxo metabolites of 1α,25$(OH)_2D_3$ and two of its potent synthetic analogs (1α,25-$(OH)_2$-16-ene-D_3 and 1α,25-$(OH)_2$-20-*epi*-D_3) using a rat kidney perfusion system. We have found that the 24-oxo metabolites of both 1α,25$(OH)_2D_3$ and its analogs have either the same or greater antiproliferative activity against various cancer cells as their parental compounds. Notably, two cell lines (DU-145 (prostate cancer) and MDA-MB-436 [breast cancer]) that were extremely resistant to the antiproliferative effects of vitamin D_3 analogs displayed greater sensitivity towards the 24-oxo metabolite of the vitamin D_3 analog. Similarly, the 24-oxo metabolites had the capacity to induce differentiation and apoptosis and to diminish the proportion of cells in S phase. Most interestingly, while the analog 1α,25$(OH)_2$-20-*epi*-D_3 induced expression of BRCA1 in MCF-7 breast cancer cells; its 24-oxo metabolite dramatically suppressed BRAC1 expression. Thus, we have shown for the first time that the various biological activities produced by the hormone 1α,25$(OH)_2D_3$ and some of its analogs may represent a combination of actions by the hormone 1α,25$(OH)_2D_3$ and its natural 24-oxo metabolites. J. Cell. Biochem. 66:413–425, 1997.

Key words: vitamin D_3 analogs; 24-oxo metabolites; growth inhibition; differentiation; apoptosis

A common goal of cancer therapy is restoration of normal growth control in transformed tissues. One area that has been intensively studied in recent years is biological modifiers of cancer growth which are designed to retard proliferation [Novichenko et al., 1995], to induce differentiation of these cells to a quiescent, nondividing stage [Liu et al., 1994; Samid et al., 1993], and/or to promote cell death in malignant or premalignant cells [Welsh et al., 1994; Li et al., 1995]. One of these potential biological modifiers is the seco-steroid hormone 1α,25 dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) [Niles, 1995; Mangelsdorf et al., 1995] which is a key regulator of calcium homeostasis. It has also been found to have effects on the growth and differentiation of many normal and malignant tissues. This seco-steroid initiates genomic responses through a specific nuclear vitamin D_3 receptor (VDR) acting as a ligand-inducible transcription factor which in turn interacts with a vitamin D_3 response element (VDRE) contained within the promoter/enhancer region of target genes and thereby regulates specific gene transcription. These responses include the inhibition of proliferation and induction of differentiation of various tis-

Abbreviations: CDKI, cyclin-dependent kinase inhibitor; ED_{50}, estimated dose; FACS, fluorescence activated cell sorter; PI, propidium iodide; VDR, vitamin D receptor; VDRE, vitamin D response element; 1α,25$(OH)_2$, vitamin D_3.

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sue types, including cancerous and precancerous cells. For example, $1\alpha,25(OH)_2D_3$ can inhibit the growth and/or induce differentiation in vitro of cancer cells from the human hematopoietic system, breast, colon, skin, brain, and prostate [Norman et al., 1990; Jung et al., 1994; Brenner et al., 1995; James et al., 1994; Wali et al., 1995; Thomas et al., 1994; Shabahang et al., 1994; Yu et al., 1995; Naveilhan et al., 1994; Skowronski et al., 1995; Feldman et al., 1995].

Several genes have been identified that contain a VDRE within their promoter/enhancer region. An example of a specific, genomic effect of $1\alpha,25(OH)_2D_3$ includes cell cycle arrest in G_1. Many factors can lead to a cell cycle arrest, but the cyclin-dependent kinase inhibitors (CDKIs) known as $p21^{(waf1)}$ and $p27^{(kip1)}$ are pivotal to this process; the $p21^{(waf1)}$ gene contains a VDRE within its promoter region [Liu et al., 1996b]. We and others have demonstrated both transcriptional and translational mechanisms for increased expression of $p21^{(waf1)}$ and $p27^{(kip1)}$ in response to $1,25(OH)_2D_3$ in the HL-60 and U-937 myeloid leukemia cell lines and the LNCaP prostate cancer cell line [Hengst and Reed, 1996; Wang et al., 1996; Campbell et al., in press].

One major focus of research in the field of vitamin D_3 and cancer has been to identify analogs of $1,25(OH)_2D_3$ that have prominent antiproliferative effects against cancer cells without resulting in lethal hypercalcemia when administered in vivo at pharmacologically active doses. The overwhelming majority of analogs examined thus far have been derived by chemical synthesis. Within a cell, $1,25(OH)_2D_3$ readily undergoes metabolism along several different metabolic pathways, as described recently in our previous paper [Siu-Caldera et al., 1995]. All of these pathways were thought to be catabolic, and the intermediate metabolites were believed to have little or no biological activity.

Recently, we have isolated a major natural intermediate of a potent precursor analog ($1\alpha,25(OH)_2$-16-ene-D_3) formed through the C-24 oxidation metabolic pathway using a rat kidney perfusion biotransformation system [Caldera et al., 1996]. This intermediary metabolite, $1\alpha,25(OH)_2$-24-oxo-16-ene-D_3, is significantly resistant to further metabolism and therefore accumulates. This metabolite shares approximately the same level of potency as its parental analog ($1\alpha,25(OH)_2$-16-ene-D_3) in inhibiting proliferation and inducing differentiation of the human leukemic cell line RWLeu-4 and in transactivating a VDRE reporter construct [Caldera et al., 1996]. Most interestingly, the 24-oxo metabolite had reduced calcemic activity as compared to its parental analog [Lemire et al., 1994]. Therefore, the 24-oxo metabolites appear to allow a separation of the antiproliferative genomic effects of vitamin D_3 compounds from their hypercalcemic side effects.

We have now undertaken a more comprehensive study of the activities of these 24-oxo metabolites compared to their precursor analogs. The $1\alpha,25(OH)_2D_3$ and two potent analogs (containing either a 16-ene or a 20-*epi* modification to the side chain) were compared to their corresponding 24-oxo metabolites for activity against cell lines of three types of cancers: human myeloid leukemia (HL-60), breast cancer (MCF-7 and MDM-MB-436), and prostate cancer (LNCaP and DU-145).

Initially, we compared the abilities of the vitamin D_3 analogs and their corresponding 24-oxo metabolite to inhibit clonal proliferation of the cancer cells. Furthermore, their effects on the cell cycle, differentiation, and apoptosis of the cancer cells were studied. We particularly focused on one of the breast cancer cell lines (MCF-7), examining modulation of its $p21^{(waf1)}$ and $p27^{(kip1)}$ and the expression of BAX, an apoptosis-inducing protein.

Two other hormonally regulated proteins possibly important in the growth regulation of normal tissue were studied: the cell-surface adhesion molecule E-cadherin and the recently identified breast cancer susceptibility protein, BRCA1 [Miki et al., 1994]. These putative tumor suppressors are commonly mutated in several types of tumors; for example, the BRCA1 gene is frequently mutated in breast and ovarian cancers [Marquis et al., 1995]. These cancers are also often associated with altered expression of E-cadherin. For example, 50% of metastatic breast cancers have downregulated expression of E-cadherin [Berx et al., 1995]. Deregulated expression of both proteins is associated with a more transformed, less differentiated phenotype [Sitonen et al., 1996; Eisinger et al., 1996].

MATERIALS AND METHODS

Cells

All cell lines were obtained from ATCC (Rockville, MD) and maintained according to their

recommendations. HL-60 cells are leukemic promyelocytes isolated from a patient with acute myeloid leukemia. MCF-7 and MDA-MB-436 were developed from pleural effusions of patients with metastatic adenocarcinoma of the breast. LNCaP has been derived from a lymph node metastasis from a patient with hormonally refractory prostate cancer. DU-145 was established from a prostate cancer metastatic to the brain. Salient features of these cell lines are summarized in Table I.

Vitamin D_3 Analogs and Their 24-Oxo Metabolites

The concentration of all compounds was determined via absorbance spectroscopy at 265 nm. 1α,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-16-ene-D_3 were synthesized at Hoffmann LaRoche, (Nutley, NJ), and 1α,25-$(OH)_2$-20-*epi*-D_3 was synthesized at Leo Pharmaceuticals (Ballerup, Denmark). The 24-oxo metabolites of 1α,25-$(OH)_2$-D_3 and its two analogs were produced using the kidney perfusion system, as previously described [Caldera et al., 1996]. All the compounds were then kept in stock vials at 10^{-3} M in ethanol at −20°C in the dark. For experimental use, they were diluted in media with 10% fetal calf serum. The compounds were assigned number codes as follows: cpd. 1, 1α,25-$(OH)_2$-D_3; cpd. 1a, 1α,25-$(OH)_2$-24-oxo-D_3; cpd. 2, 1α,25-$(OH)_2$-16-ene-D_3; cpd. 2a, 1α,25-$(OH)_2$-24-oxo-16-ene-D_3; cpd. 3, 1α,25-$(OH)_2$-20-*epi*-D_3; cpd. 3a, 1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3.

Effect of Vitamin D_3 Analogs and Their 24-Oxo Metabolites on Clonal Growth of Cancer Cells in Soft Agar

Potency of vitamin D_3 analogs and their 24-oxo metabolites was determined by extensive dose-response studies in soft agar. Target cancer cells from 80% confluent cultures were plated into 24-well, flat-bottom plates using a two-layer soft agar system with a total volume of 400 µl, as described previously [Munker et al., 1986]. The cells were maintained in their respective media. The feeder layer was prepared with agar (1%) that had been equilibrated to 42°C. Prior to addition of this layer to the plate, the vitamin D_3 analogs and metabolites were pipetted into the wells. After 10 days (HL-60) and 14 days (breast and prostate) of growth in soft agar, the colonies (≥50 cells) were counted with an inverted microscope. All experiments were done at least three times in triplicate dishes per experimental point.

Mechanism of Inhibition

Effect of vitamin D_3 analogs on cell cycle and cell cycle–related proteins. The vitamin D_3–sensitive cell lines HL-60, MCF-7, and LNCaP were exposed to either analog or metabolite, and the cell cycle distribution was determined. DNA was stained with propidium iodide (PI) after a total of 5×10^5 subconfluent, exponentially proliferating cells were either cultured with the vitamin D_3 compounds (10^{-7} M for 3 days) or left untreated (control). Total cells, both in the media and remaining adherent, were harvested, washed, resuspended in PBS, and stained with trypan blue, and viable and nonviable cells were counted. The cells were adjusted to a final concentration of 1×10^6 viable cells/ml and fixed in a 2:1 (vol/vol) ratio in chilled methanol overnight prior to staining with PI in the presence of RNAse One (Promega, Madison, WI). Cell cycle distribution was determined on a Becton-Dickinson (Braintree,

TABLE I. Summary of Key Features of Target Cancer Cell Lines*

	Leukemia	Prostate cancer		Breast cancer	
Tissue type	HL-60	LNCaP	DU-145	MCF-7	MDA-MB-436
Expression VDR	wt	wt	wt	wt	wt
Sensitive to clonal inhibition by 1α,25-$(OH)_2$-D_3	[a]	[b]	[c]	[a]	[c]
Expression p53	ko	wt	ko	wt	NI
Expression Rb	wt	wt	ko	wt	NI
Sensitive to sex hormone	NI	[d]	[e]	[d]	[e]

*ko, functional knockout of gene; NI, not investigated; wt, wild-type expression.
[a]Extreme sensitivity to 1α,25-$(OH)_2$-D_3.
[b]Moderate sensitivity to 1α,25-$(OH)_2$-D_3.
[c]Insensitive to 1α,25-$(OH)_2$[D_3.
[d]Sensitive to androgen (LNCaP) or estrogen (MCF-7).
[e]Insensitive to androgen (LNCaP) or estrogen (MCF-7).

MA) FACScan Flow Cytometer and CellFIT Cell-Cycle Analysis software.

Expression of $p21^{(waf1)}$ in HL-60 and MCF-7 was examined after 48 h exposure to the precursor analog or metabolites (10^{-7} M). In a similar manner, $p27^{(kip1)}$ was examined in MCF-7 after 0–4 days exposure to the same vitamin D_3 compounds. Control and test cultures of both HL-60 and MCF-7 cells were maintained for the same durations at parallel cell numbers. Cellular lysates from cultures were subjected to SDS-PAGE. Briefly, extracts from 1.5×10^6 cells were boiled in sample buffer for 5 min and loaded onto a 12.5% SDS-polyacrylamide gel. After electrophoresis at 150 V, the proteins were transferred to Immobion-P (Millipore, Bedford, MA) membrane, blocked with Tris-buffered saline containing Tween 20 (0.1%) and gelatin (1%) at pH 7.5 for 1 h, and then incubated with antibodies to either $p21^{(waf1)}$ (Oncogene Research Products) or $p27^{(kip1)}$ (Santa Cruz Biotechnology, Santa Cruz, CA) [Upadhyay et al., 1995; Fang et al., 1996]. The proteins were detected using an ECL chemiluminescence system (Amersham Life Sciences, Arlington Heights, IL). To ensure even loading of proteins, nonspecific bands were compared, and the membrane was stained afterwards with Ponceau S. Densitometry was performed on bands to quantify the changes in detected protein.

Effect of vitamin D_3 analogs on differentiation. Induction of differentiation was measured by the modulation of surface expression of CD 11b on HL-60 cells, secretion of prostate specific antigen (PSA) by LNCaP cells, and modulation of E-cadherin and BRCA1 in MCF-7 cells. Expression of the surface marker CD 11b was measured by direct immunofluorescence. Approximately 5×10^5 cells were incubated with a saturating concentration of murine CD 11b antibody (Carpenteria, CA) for 45 min on ice, followed by incubation with a goat anti-mouse FITC-conjugated secondary antibody. A FITC-conjugated isotype control was used. Immunofluorescence was analyzed with a Becton-Dickinson FACScan Flow Cytometer using LYSIS II software.

Changes in PSA were measured by plating LNCaP cells (1×10^5) into six-well dishes in 3 ml of media with either cpd. 2 (1α,25-$(OH)_2$-16-ene-D_3), cpd. 2a (1α,25-$(OH)_2$-24-oxo-16-ene-D_3) (10^{-7} M), or media alone (control). Media were harvested at day 4, and PSA levels were measured by a TANDEM-E ELISA method; viable cells were counted to normalize PSA levels.

Modulation of expression of the BRCA1 and E-cadherin proteins was measured by Western blot analysis as indicated above, using antibodies for E-cadherin (Transduction Laboratories, Lexington, CA) and BRCA1 (Santa Cruz Biotechnology) [Tamm et al., 1994; Chen et al., 1995].

Effect of vitamin D_3 analogs on apoptosis. HL-60, MCF-7, and LNCaP cells were exposed to the vitamin D_3 analog and its 24-oxo metabolite (10^{-7} M). Fresh media and vitamin D_3 compounds were added on day 2 of culture, and at that time any detached cells were placed back in the test cultures. On day 4, DNA fragmentation was measured as described previously [Li and Daryzynkiewicz, 1995]. Briefly, total cells, both in the media and those remaining adherent to the plastic dishes, were harvested and fixed in 1% methanol-free formaldehyde for 15 min and washed in phosphate buffered saline (PBS). The cell concentration was corrected to 1×10^6 cells/ml, and these cells were fixed in 5 ml of 70% ethanol. Single- and double-strand DNA breaks were labeled with bromodeoxyuridine triphosphate (BrD-UTP) for 40 min at 37°C with terminal transferase (Boehringer-Mannheim, Indianapolis, IN). The cells were permeabilized with a 0.3% solution of Triton-X 100 in 0.5% bovine serum albumin (BSA)/PBS. Cells that had breaks in DNA were tagged by the incorporation of BrDU and were identified with a FITC-conjugated anti-BrDU antibody. Cells were stained with PI for 30 min, and green fluorescence was measured by FACS analysis at 510–550 nm. As a positive control, cells were treated with etoposide (50 µg/ml for 2 days).

Modulation of expression of the apoptosis-associated protein BAX was measured by Western blot analysis as indicated above using antibody for BAX (Santa Cruz Biotechnology) [Elstner et al., 1996].

RESULTS

Clonal Inhibition of Proliferation of Leukemic, Breast, and Prostate Cancer Cells Mediated by Vitamin D_3 Analogs and Their 24-Oxo Metabolites

Three pairs of vitamin D_3 compounds and their corresponding 24-oxo metabolites were examined for their effects on the clonal proliferation of a leukemic, two breast, and two prostate

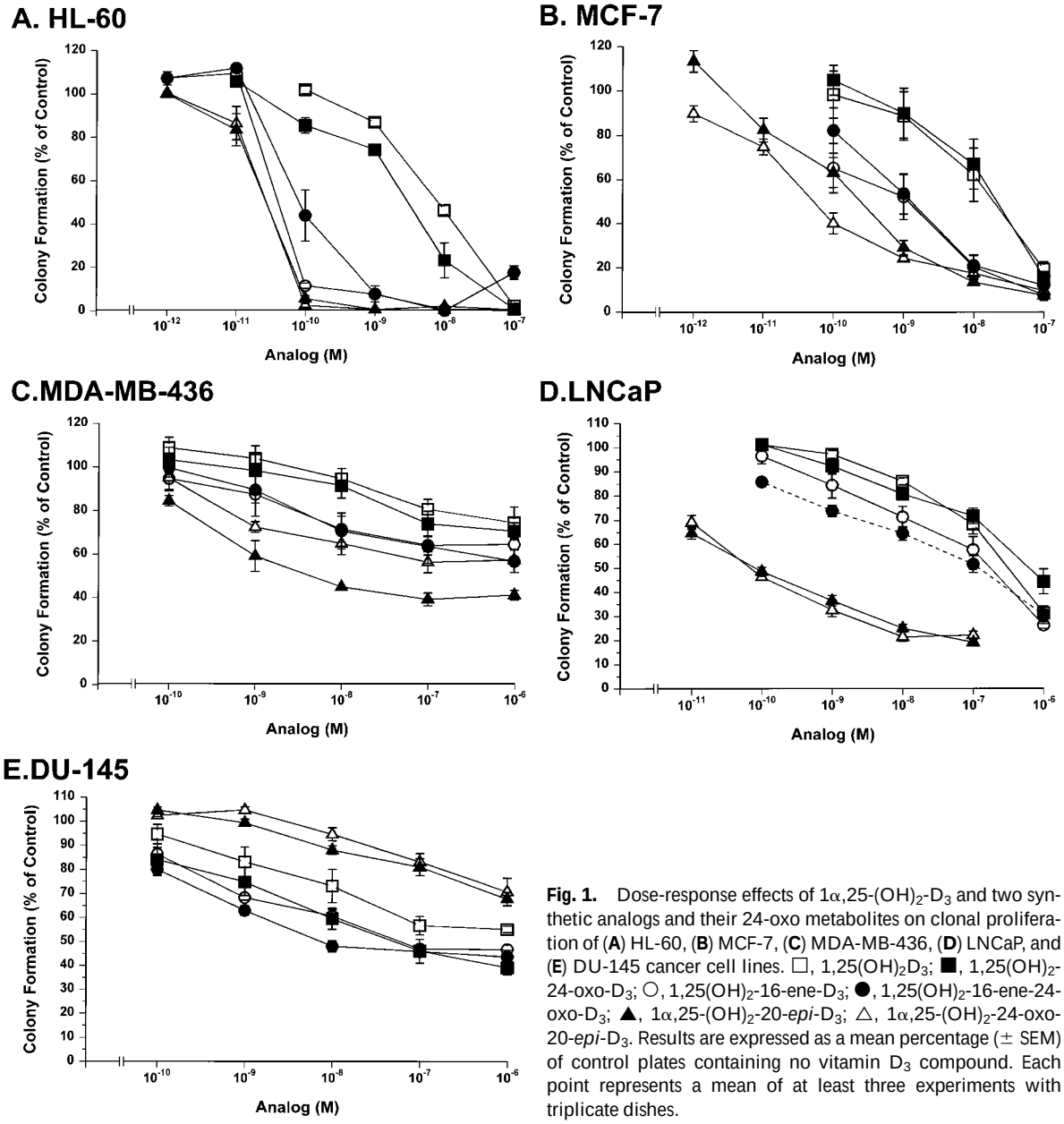

Fig. 1. Dose-response effects of 1α,25-$(OH)_2$-D_3 and two synthetic analogs and their 24-oxo metabolites on clonal proliferation of (**A**) HL-60, (**B**) MCF-7, (**C**) MDA-MB-436, (**D**) LNCaP, and (**E**) DU-145 cancer cell lines. □, 1,25$(OH)_2D_3$; ■, 1,25$(OH)_2$-24-oxo-D_3; ○, 1,25$(OH)_2$-16-ene-D_3; ●, 1,25$(OH)_2$-16-ene-24-oxo-D_3; ▲, 1α,25-$(OH)_2$-20-*epi*-D_3; △, 1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3. Results are expressed as a mean percentage (± SEM) of control plates containing no vitamin D_3 compound. Each point represents a mean of at least three experiments with triplicate dishes.

cancer cell lines (Fig. 1A–E). The estimated dose required to inhibit clonal proliferation by 50% (ED_{50}) is summarized in TableII.

1α,25-$(OH)_2$-D_3 and its 24-oxo derivative. The natural hormone and its 24-oxo metabolite were the least potent of the three pairs of compounds. The ED_{50} was approximately the same for cpd. 1 (1α,25-$(OH)_2$-D_3) or cpd. 1a (1α,25-$(OH)_2$-24-oxo-D_3) (Table II). For example, the ED_{50}s for HL-60 were 10 nM (cpd. 1) and 3 nM (cpd. 1a) (Fig. 1A; Table II). One remarkable finding was that the perniciously resistant prostate cancer cell line DU-145 (ED_{50} > 1,000 nM for 1α,25-$(OH)_2$-D_3) displayed significant sensitivity to the 24-oxo metabolite of 1α,25-$(OH)_2$-D_3 (ED_{50}, 50 nM) (Fig. 1E; Table II).

16-ene-vitamin D_3 analog and its 24-oxo derivative. The 1α,25-$(OH)_2$-16-ene-D_3 has been shown previously to be more potent than 1α,25-$(OH)_2$-D_3 [Jung et al., 1994], and the present data reflect this observation, as nearly all the ED_{50} values for the 16-ene analog are lower than those for 1α,25-$(OH)_2$-D_3 (Table II). For example, with the HL-60 cell line as a target, the ED_{50} value for cpd. 1 (1α,25-$(OH)_2$-D_3) was

TABLE II. Clonal Inhibition of Leukemic, Breast, and Prostate Cancer Cells by Vitamin D_3 Compounds and Their 24-Oxo Metabolites*

Compound	HL-60	LNCaP	DU-145	MCF-7	MDA-MB-436
1. $1,25(OH)_2D_3$	10	300	>1,000	20	>1,000
1a. $1,25(OH)_2$-24-oxo-D_3	3	650	50	20	>1,000
2. $1,25(OH)_2$-16-ene-D_3	0.05	200	50	1.5	>1,000
2a. $1,25(OH)_2$-16-ene-24-oxo-D_3	0.1	150	8	1.5	>1.000
3. $1,25(OH)_2$-20-epi-D_3	0.02	0.1	>1,000	0.05	>1,000
3a. $1,25,(OH)_2$-20-epi-24-oxo-D_3	0.02	0.1	>1,000	0.25	5

*Extensive (≥3 experiments done in triplicate plates) dose-response (10^{-11} to 10^{-6} M) studies were performed with each of the vitamin D_3 analogs and their 24-oxo metabolites. Dose-response curves were drawn, and the dose (nM) required to inhibit clonal formation by 50% (ED_{50}s) was interpolated.

10 nM, and for cpd 2 (1α,25-$(OH)_2$-16-ene-D_3) it was 0.05 nM (Fig. 1A). Comparable potency was observed between cpd. 2 (1α,25-$(OH)_2$-16-ene-D_3) and its oxo-metabolite 2a (1α,25-$(OH)_2$-24-oxo-16-ene-D_3) for MCF-7 cells ($ED_{50} \approx 1.5$ nM) (Fig. 1B) and for LNCaP cells (ED_{50}, 200 nM for cpd. 2 and 150 nM for cpd. 2a). Cpd. 2 was more potent against HL-60 cells, while its 24-oxo metabolite (cpd. 2a) was more potent against DU-145 cells (Table II).

20-*epi*-vitamin D_3 analog and its 24-oxo derivative. We [Elstner et al., 1994, 1995] as well as others [Dilworth et al., 1994; Binderup et al., 1991] have shown that the 20-*epi* orientation of the side chain markedly enhanced the antiproliferative potency of 1α,25$(OH)_2D_3$ against leukemic, breast, and prostate cancer cells. We therefore included the 24-oxo metabolite of 1α,25-$(OH)_2$-20-*epi*-D_3. Compounds 3 (1α,25-$(OH)_2$-20-*epi*-D_3) and 3a (1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3) were of comparable potency, and they were at least, if not more, potent than the 16-ene modified compounds (Table II).

We discovered that the 24-oxo metabolite of 20-epi-D_3 (cpd. 3a) has prominent antiproliferative activity against MDA-MB-436 breast cancer cells cells (ED_{50}, 5 nM) (Fig. 1C). This finding is particularly notable because MDA-MB-436 breast cancer cells were previously found to be entirely resistant to all vitamin D_3 compounds including the non–24-oxo analogs examined in this study [Elstner et al., 1995].

Potential Pathway of Clonal Inhibition

Analysis of the cell cycle and expression of $p21^{(waf1)}$ and $p27^{(kip1)}$. Matched pairs of potent vitamin D_3 analogs and their corresponding 24-oxo metabolites were cultured with the target cell lines HL-60, LNCaP, and MCF-7 for 3 days. The HL-60 and MCF-7 cells were exposed to cpd. 3 (1α,25-$(OH)_2$-20-*epi*-D_3) and cpd. 3a (1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3), and LNCaP was exposed to cpd. 2 (1α,25-$(OH)_2$-16-ene-D_3) and cpd. 2a (1α,25-$(OH)_2$-24-oxo-16-ene-D_3).

The changes in the cell cycle after exposure to these combinations of potent vitamin D_3 analogs and their corresponding 24-oxo metabolites are shown in Table III. The data show that exposure to these compounds increased the proportion of cells in G_0/G_1; the proportion in S and G_2/M phases decreased for each of the cell lines after their exposure to the analogs and their metabolites. For example, HL-60 cells cultured with cpd. 3 and cpd. 3a increased the proportion of G_0/G_1 cells from 48 ± 0.1% (untreated cells) to 67 ± 2.1% and 65 ± 0.2%, respectively.

The $p21^{(waf1)}$ and $p27^{(kip1)}$ are CDKIs that bind to cyclin-cyclin-dependent kinase complexes and decrease kinase activity. Vitamin D_3 mediated increases in expression of $p21^{(waf1)}$ which peaked at 48 h of exposure [Munker et al., 1996]. Therefore, cpd. 3 and cpd. 3a (10^{-7} M) were cultured for 48 h with HL-60 and MCF-7 cells (Fig. 2). Levels of expression of $p21^{(waf1)}$ in HL-60 cells increased by 260% and 150% after exposure to cpd. 3 and 3a, respectively. Levels of $p21^{(waf1)}$ did not change in MCF-7 cells exposed to either compound. Furthermore, under the same experimental conditions, levels of $p27^{(kip1)}$ did not change after 96 h exposure of MCF-7 cells to either cpd. 3 and or 3a (data not shown).

Effect of vitamin D_3 analogs on differentiation. To examine the differentiation-inducing effects that may be associated with inhibition of proliferation, we analyzed the effect of the same matched pairs of vitamin D_3 analogs and the corresponding 24-oxo metabolite as used for the cell cycle analysis. The surface marker CD 11b is associated with differentiation of HL-60 cells. Treatment of HL-60 with either cpd. 3 or 3a resulted in an equally significant

TABLE III. Cell Cycle Distribution by Mediated Vitamin D_3 Analogs and Their 24-Oxo Metabolites*

Vitamin D_3 compounds	HL-60 (%)		MCF-7 (%)		LNCaP (%)	
Control	G_0/G_1	49 ± 0.1	G_0/G_1	38 ± 1.5	G_0/G_1	66 ± 1.5
	S	37 ± 0.3	S	35 ± 0.8	S	27 ± 0.7
	G_2/M	14 ± 0.3	G_2/M	26 ± 0.9	G_2/M	10 ± 1.2
2. 1α,25-$(OH)_2$-16-ene-D_3					G_1/G_1	77 ± 0.7
					S	18 ± 0.9
					G_2/M	5 ± 1.1
2a. 1α,25-$(OH)_2$-24-oxo-16-ene-D_3					G_0/G_1	78 ± 1.0
					S	13 ± 1.1
					G_2/M	8 ± 0.6
3. 1α,25-$(OH)_2$-20-*epi*-D_3	G_0/G_1	67 ± 2.1	G_0/G_1	52 ± 1.6		
	S	26 ± 0.4	S	26 ± 1.4		
	G_2/M	6 ± 1.3	G_2/M	21 ± 1.3		
3a. 1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3	G_0/G_1	65 ± 0.2	G_0/G_1	51 ± 0.4		
	S	26 ± 0.5	S	27 ± 1.0		
	G_2/M	9 ± 0.4	G_2/M	22 ± 0.7		

*Cells (HL-60, leukemia cells; MCF-7 and LNCaP breast and prostate cancer cells) were plated in triplicate wells and grown in the presence or absence (control) of the test compounds. After 3 days, the cell cycle distribution was measured (Materials and Methods).

increase in CD 11b expression with both compounds (data not shown). Prostate-specific antigen (PSA) is a secreted protease of the normal prostate which has also been used as a prostate-specific tumor marker. Investigators have suggested that it may also be a differentiation marker of normal prostate development [Wang et al., 1996]. Similarly, LNCaP was cultured with either cpd. 2 or 2a (10^{-7} M, 4 days) and produced an equal increase in PSA with each compound (approximately 70%) (data not shown). Thus, both vitamin D_3 analogs and their 24-oxo metabolites were equally able to induce differentiation of HL-60 and LNCaP cells.

E-cadherin is a cell surface adhesion molecule that is essential for maintaining the cell-cell adhesion system, and its decreased expression has been associated with metastatic disease [Sitonen et al., 1996]. The breast cancer cell line MCF-7 showed an approximately 180% increase in the expression of E-cadherin after 4 days exposure (10^{-7} M) to either cpd. 3 or 3a (Fig. 3).

The BRCA1 protein has a putative tumor suppressor role in breast and ovarian cancer cells, although the exact function of this protein is unclear [Jensen et al., 1996]. One of the most interesting findings of the present study is the inverse modulation of BRCA1 protein in response to either cpd. 3 or 3a (Fig. 4). The parent analog, cpd. 3 (1α,25-$(OH)_2$-20-*epi*-D_3) (10^{-7} M), induced a modest increase of BRCA1 expression (130% at 48 h), and this was sustained at 96 h of culture. However, the corresponding 24-oxo metabolite, cpd. 3a (1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3) (10^{-7} M), demonstrated the opposite behavior, dramatically decreasing BRCA1 levels by 25% at 48 h and 75% by 96 h of culture.

Effect of vitamin D_3 analogs and 24-oxo metabolites on apoptosis. Cells arrested in their progression through the cell cycle may undergo one of several fates, including apoptosis, which was investigated in a similar manner to the cell cycle analysis. Three target cell lines (MCF-7, HL-60, and LNCaP) were exposed to the same vitamin D_3 analogs or their 24-oxo metabolites used for the cell cycle analysis (10^{-7} M, 4 days). Only MCF-7 cells, not HL-60 or LNCaP cells, underwent apoptosis in response to either the analog or metabolite as detected by FACS analysis of DNA single- and double-strand breaks (Fig. 5). The level of apoptosis in MCF-7 cells was approximately 9.5 ± 0.3% with cpd. 3, but its 24-oxo metabolite (cpd. 3a) significantly increased (doubled) ($P \leq 0.02$) the proportion of apoptotic cells to 23 ± 1.6%. Enhanced BAX expression has been associated with apoptosis. Correlated with the induction of apoptosis of MCF-7, exposure of these cells to cpd. 3 increased by 190% (48 h) and 150% (96 h) the expression of BAX as determined by Western blot. The 24-oxo metabolite (cpd. 3a) increased by 150% (96 h) the levels of BAX (Fig. 6).

DISCUSSION

In this study, we have compared the biological effects of 1α,25-$(OH)_2$-D_3 and two of its syn-

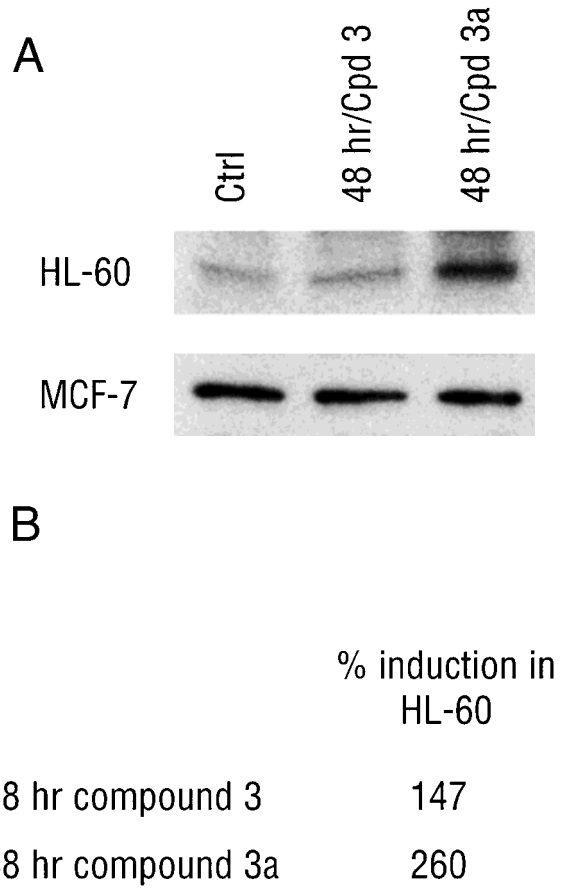

Fig. 2. Modulation of p21$^{(waf)}$ protein expression in HL-60 and MCF-7 cells. HL-60 and MCF-7 cells were treated with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated time periods or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and p21$^{(waf)}$ was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

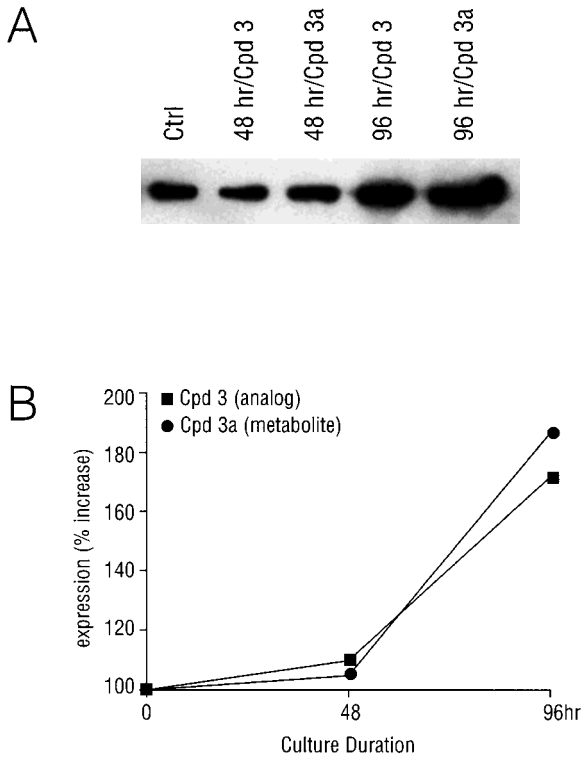

Fig. 3. Differentiation of MCF-7 cells as measured by modulation of E-cadherin protein expression. E-cadherin expression by MCF-7 cells was measured after treatment with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated durations or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and E-cadherin was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

thetic analogs and their corresponding 24-oxo metabolites on leukemic, breast, and prostate cancer cell lines. The initial analysis of activity utilized the exquisitely sensitive soft agar clonogenic assay which examines the clonal capacity of single cells to undergo at least six divisions. The 24-oxo metabolites generally had the same activity as their parental analogs. However, several exceptions are very interesting. The DU-145 cell line is resistant to growth inhibition by most vitamin D$_3$ analogs [Campbell et al., in press]. In contrast, the 24-oxo metabolite of 1α,25-(OH)$_2$-D$_3$ (cpd. 1a [1α,25(OH)$_2$-24-oxo-D$_3$]) but not 1α,25(OH)$_2$-D$_3$ markedly inhibited clonal growth of these cells (ED$_{50}$, 50 nM). Similarly, the breast cancer cell line MDA-MB-436 is resistant to growth inhibition by most vitamin D$_3$ analogs [Elstner et al., 1995]. However, cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) but not cpd. 3 [1α,25(OH)$_2$-20-*epi*-D$_3$] inhibited the clonal growth of these cells (ED$_{50}$, 5 nM). The ability of these two 24-oxo metabolites to inhibit the clonal growth of other cancer cell lines known to be resistant to most vitamin D$_3$ compounds is now being explored.

Cell cycle analysis of the three vitamin D$_3$–sensitive cell lines revealed that the precursor vitamin D$_3$ analogs and their 24-oxo metabolites had similar potencies to induce a G1 arrest of cancer cells. Likewise, they had similar abilities to induce differentiation, as measured by an increased expression of CD 11b on HL60 cells and increased secretion of PSA by LNCaP cells.

Apoptosis did not significantly increase in either HL-60 or LNCaP cells cultured with either the vitamin D$_3$ analogs or their 24-oxo metabolites. However, the breast cancer cell

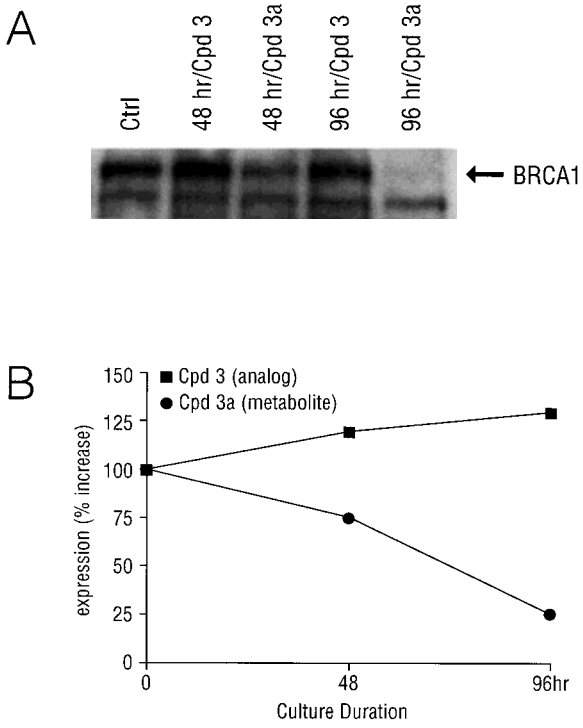

Fig. 4. Modulation of BRCA1 protein expression in MCF-7 cells. BRCA1 expression by MCF-7 cells was measured after treatment with either cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) at 10^{-7} M for the indicated durations or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and BRCA1 was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

line MCF-7 doubled its level of apoptosis (23 ± 1.6%) when cultured with the 24-oxo metabolite of cpd. 3 (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$). Taken together, the data on clonal inhibition, cell cycle arrest, differentiation, and apoptosis indicate that the 24-oxo metabolites are at least as potent as their precursor vitamin D$_3$ compounds. Therefore, this study clearly shows that metabolites of 1α,25-(OH)$_2$-D$_3$ can be active, and these findings contradict for the first time the previous conventional wisdom. Furthermore, the data also suggest that the 24-oxo metabolites may possess unique antiproliferative characteristics. The full spectrum of these features is now being explored.

To examine further the molecular effects of the compounds, we examined the effect of cpd. 3 and cpd. 3a on levels of expression of several CDKIs which are integrally associated with cell cycle arrest, apoptosis, and differentiation. Expression of p21$^{(waf1)}$ has been reported to be upregulated by 1α,25(OH)$_2$-D$_3$ in HL-60 cells [Munker et al., 1996]. We confirmed this finding using cpd 3 (1α,25(OH)$_2$-24-oxo-D$_3$) and also found that the 24-oxo metabolite could also increase the levels of expression of p21$^{(waf1)}$ in HL-60 cells. By contrast, the MCF-7 breast cancer cells did not increase their expression of p21$^{(waf1)}$ after culture with either the vitamin D$_3$ analog or its 24-oxo metabolite. Previous studies have suggested that induction of expression of p21$^{(waf1)}$ can have an apoptosis-inhibitory role during the process of differentiation [Wang et al., 1996]. Perhaps the apoptosis is induced by vitamin D$_3$ compounds in MCF-7 cells because p21$^{(waf1)}$ is not modulated in these cells. Likewise, expression of p27$^{(kip1)}$ was not increased by the vitamin D$_3$ compounds in these cells, although we [Siu-Caldera et al., 1995] and others [Hengst and Reed, 1996; Campbell et al., in press] have found enhanced expression of this CDKI in other cells types cultured with vitamin D$_3$ compounds. Another key regulatory protein in the process of apoptosis is BAX. It was slightly upregulated after treatment with either 1α,25(OH)$_2$-20-*epi*-D$_3$ or 1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$. Taken together, we still do not have a clear understanding of the molecular events responsible for the growth inhibition of cancer cells mediated by the vitamin D$_3$ analogs and their 24-oxo metabolites. Most of our target cancer cells are inhibited in their clonal growth at the G$_1$ stage of the cell cycle by the vitamin D$_3$ compounds.

The effect of the vitamin D$_3$ analogs and their 24-oxo metabolites on expression of E-cadherin and BRCA1 was also examined, as both have importance in the development or progression of breast cancer. Murine gene-knockout studies have shown both proteins to be essential for embryonic development [Liu et al., 1996a]. E-cadherin controls growth by binding with the cytoplasmic integrin proteins. This interaction can modulate cellular proliferation by reacting internally with other proteins such as the APC tumor suppressor gene [Su et al., 1993]. Both the analog and its 24-oxo metabolite induced an equally upregulated expression of E-cadherin, which may contribute to their comparable level of cell cycle arrest of the MCF-7 cells.

The exact function of the BRCA1 protein is less well characterized. Retroviral studies have demonstrated that wild-type but not mutant forms of the BRCA1 gene inhibit growth in vitro of some breast and ovarian cancer cell lines [Holt et al., 1996]. To our knowledge, we

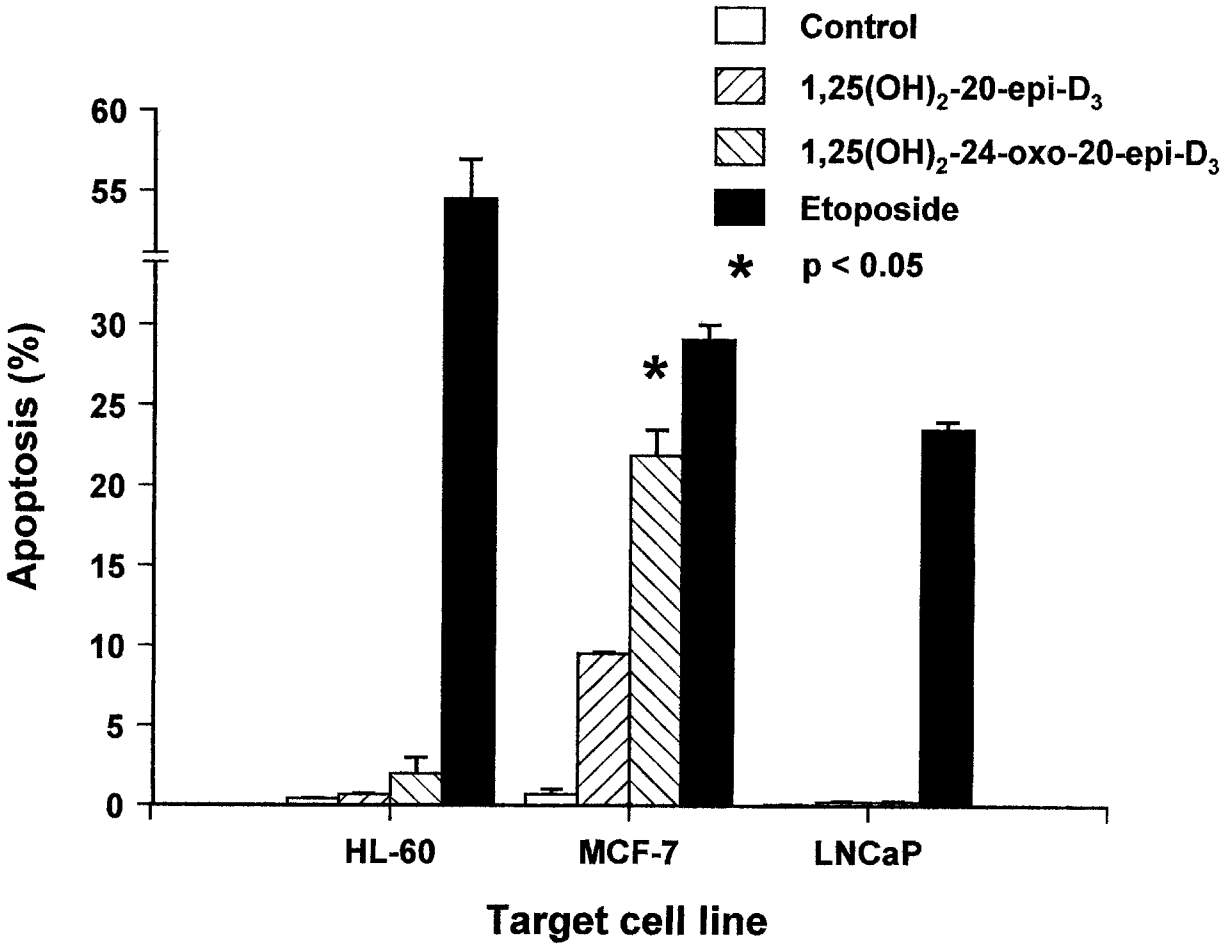

Fig. 5. Apoptosis measurements. HL-60, MCF-7, and LNCaP were exposed to cpd. 3 (1α,25(OH)$_2$-20-*epi*-D$_3$) or cpd. 3a (1α,25(OH)$_2$-24-oxo-20-*epi*-D$_3$) (HL-60 and MCF-7) and cpd. 2 (1α,25-(OH)$_2$-16-ene-D$_3$) and cpd. 2a (1α,25-(OH)$_2$-24-oxo-16-ene-D$_3$) (LNCaP) (10^{-7} M, 4 days), and apoptosis was measured as described in Materials and Methods. Each cell line was treated with 50 μg/ml etoposide as a positive control and also evaluated for apoptosis.

show for the first time that vitamin D$_3$ compounds can modulate expression of BRCA1. The modulation of this protein revealed a striking difference when comparing the vitamin D$_3$ analog and its 24-oxo metabolite; the 20-epi analog slightly increased levels of BRCA1, whereas the 24-oxo metabolite dramatically downregulated expression of BRCA1. The mechanism by which this disparate activity occurred requires further analysis. Negative regulation by vitamin D$_3$ has been demonstrated by us to be the result of posttranscriptional control of protein expression of [Tobler et al., 1988]. Others have shown it to be the result of direct transcriptional control [Peleg et al., 1993; Liu et al., 1996c]. Thus, it is not without precedent that a vitamin D$_3$ compound might have a negative regulatory role on protein expression. Perhaps the analog and its 24-oxo metabolite induce a novel conformation of the VDR, thereby allowing their interaction with different sets of VDRE. Recently, estrogen (another steroid hormone) and one of its metabolites have been shown to interact with different response elements. This allows the parental hormone and the metabolite to exert differing cellular and tissue-specific responses [Yang et al., 1996]. Potentially, vitamin D$_3$ or its analogs upregulate expression of BRCA1; then, by conversion to the 24-oxo metabolite, the cell may negatively regulate the same gene, possibly by upregulation of other BRCA1 negative-regulatory proteins. We are currently investigating this finding more fully.

In summary, we have compared for the first time three vitamin D$_3$ compounds and their 24-oxo metabolites and defined their biological potency against cancer cells. In general, the

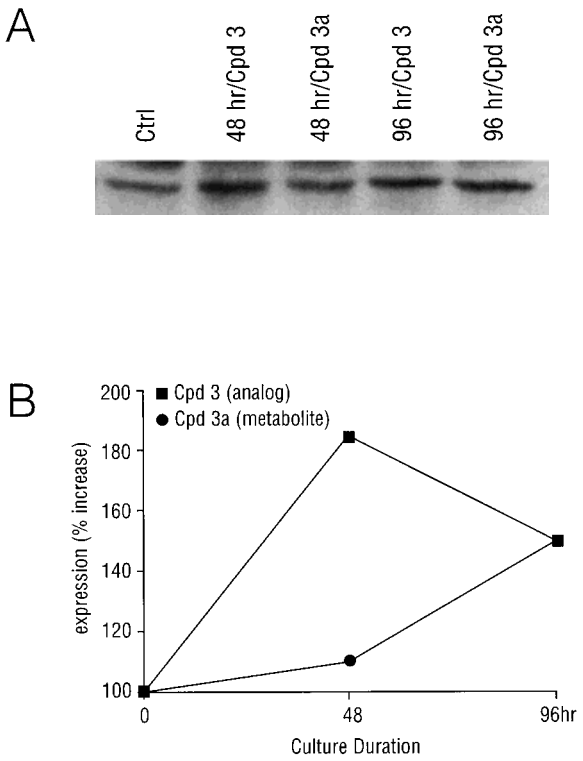

Fig. 6. Expression of BAX. BAX expression by MCF-7 cells was measured after treatment with either cpd. 3 (1α,25$(OH)_2$-20-*epi*-D_3) or cpd. 3a (1α,25$(OH)_2$-24-oxo-20-*epi*-D_3) at 10^{-7} M for the indicated durations or left untreated. **A:** Cell lysates were resolved by SDS-PAGE, and BAX was detected by Western blot analysis using the antibody described in Materials and Methods. **B:** Densitometry was performed on bands to quantify the changes in detected protein.

24-oxo metabolites, which had previously been thought to be biologically inactive, were shown to be at least as potent as their parental vitamin D_3 compounds as measured by their inhibition of cancer cell clonal proliferation, arrest of the cell cycle, and induction of differentiation and apoptosis. At the molecular level, the precursor compounds and their 24-oxo metabolites can behave in a disparate manner, as exemplified by the modulation in BRCA1 expression in breast cancer cells. Previously, we have shown that cpd. 2a (1α,25-$(OH)_2$-24-oxo-16-ene-D_3) [Lemire et al., 1994] and cpd. 3a (1α,25-$(OH)_2$-24-oxo-20-*epi*-D_3) (manuscript in preparation) have lower in vivo calcemic effects than their precursor analogs; thus, these metabolites are very attractive candidates for future therapeutic investigation.

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