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TITLE: Mechanism of Action of a Novel Analog of Vitamin D, 1α-hydroxy-24-ethyl Cholecalciferol (VD5), in Normal and Transformed Human Breast Epithelial Cells

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13. ABSTRACT *(Maximum 200 Words)*
Several epidemiological studies had suggested a correlation between increased breast cancer mortality rates and lower serum levels of vitamin D_3.The active form of vitamin D ($1,25(OH)_2D_3$) has now been well recognized as an effective suppressing agent for leukemia, breast, colon, and prostate cancers. However, due to its hypercalcemic activity it is toxic at levels that are necessary for its chemopreventive effects. Therefore, much attention has been paid to developing non-toxic analogs of vitamin D. We have been studying an analog of vitamin D, 1-hydroxy-24-ethyl Cholecalciferol (D5), for the past three years. This analog has shown antiproliferative and differentiation-inducing effects in carcinogen-transformed mouse mammary gland organ culture (MMOC) and breast cancer cells *in vitro* with little or no calcemic activity *in vivo*. D5 inhibited preneoplastic lesions and growth of carcinogen treated MMOC, but it had no effect on the growth of normal MMOC. Consequently, we proposed to transform a normal breast epithelial cell line MCF12F and to study the mechanism of action of D5 on the growth of normal *versus* transformed cell lines. MCF-12F cells were transformed with DMBA and MNU, and resulting cell lines were designated MCF-$12F_{DMBA}$ and MCF-$12F_{MNU}$, respectively. To study the growth effects of D5 on these cell lines, we performed cell count, cell viability MTT assay and FACS cell cycle analysis. Our results showed that the rate of growth of the transformed cells increased five fold and there was a loss of contact inhibition in the transformed cell lines. Cell count and MTT assay showed 40-70 % growth inhibition of MCF-$12F_{DMBA}$ and MCF-$12F_{MNU}$ with D5 treatment, while no effect was observed on the normal cells. Likewise, D5 treatment arrested the MCF-$12F_{DMBA}$ and MCF-$12F_{MNU}$ cells in G-1 phase of the cell cycle. While D5 treated MCF-12F were not different from control. In conclusion, D5 is effective in suppressing growth of carcinogen-transformed MMOC and MCF-$12F_{DMBA}$ and MCF-$12F_{MNU}$ cells, while it does not affect the growth or morphology of normal MMOC or normal breast epithelial cells. This suggests a selective effect of D5 on cancer cells. Hence, D5 can be developed as a promising chemopreventive agent. (Supported by US Army Research Materiel Command under DAMD17-01-1-0272).

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INTRODUCTION

Breast cancer is the second major cause of cancer deaths among women [1]. The active form of vitamin D ($1,25(OH)_2D_3$ or VD_3) has been well recognized as an effective suppressing agent for leukemia and breast, colon, and prostate cancers [2,3]. Several *in vitro* studies support the role of VD_3 as an antiproliferator and inducer of differentiation in breast cancer cells [4]. However, due to its hypercalcemic activity it is toxic at levels that are necessary for its chemopreventive effects. Therefore, much attention has been paid to developing analogs that lack calcium-elevating activity but possess cancer-suppressing ability of VD_3. Our lab has been studying an analog of vitamin D, 1α-hydroxy-24-ethyl cholecalciferol (D5), for the past four years. This analog has shown antiproliferative and differentiation-inducing effects in carcinogen-transformed mouse mammary gland organ culture (MMOC) [5] and steroid receptor positive (SR^+) breast cancer cells *in vitro* with little or no calcemic activity *in vivo*. Therefore, it is a good candidate for further investigations.

Although D5 has been effective in inhibiting growth of carcinogen-transformed MMOC and breast cancer cells, it does not inhibit the growth or morphology of normal MMOC and normal breast epithelial cells such as MCF-12F. This suggested a selective effect of D5 on cancer cells. However, the breast cancer cells are derived from different donors than normal cells and comparison between these cell lines can not be used to attribute selective action of D5 on transformation status of breast epithelial cells. Therefore, we proposed to transform the normal breast epithelial cells MCF12F using mammary specific carcinogens. The transformed cell lines are isogenic with the normal MCF-12F cells. This provides us with a useful model in studying mechanism of action of D5 and other potential chemopreventive agents in normal versus transformed cells. The elucidation of mechanism of D5 action will help us to determine: a) its suitability for the chemoprevention of specific types of breast carcinoma, b) its suitability for use as a prophylactic or therapeutic agent, and c) if the activity of D5 can be enhanced by using it in combination with other agents.

Previously, we have tested growth effects of D5 on normal human breast epithelial cells (MCF-12F), and observed no growth inhibition at 0.1 μM concentration. In the current study we transformed these normal cells to pre-cancerous by using two different types of carcinogens. After establishment of transformed cell lines, we compared and studied the effects of D5 on cell growth, gene expression patterns, and cell cycle progression in normal and transformed cells. Furthermore, we conducted some preliminary studies to determine possible interaction of D5 with estrogen and estrogen receptor. Studying D5 interaction with estrogen signaling can be useful in determining its possible combinations with other anti-estrogens for the prevention and/or therapy of breast cancer. The specific questions of the entire proposed study include the following:

- Does D5 selectively block cell cycle progression in transformed cells as compared to normal cells?
- Does D5 interact with the estrogen signaling pathway in the normal and transformed mammary epithelial cells?
- Does D5 have an inhibitory action on the cell invasion or the genes associated with cell invasion and metastasis?

SPECIFIC AIMS AND STATEMENT OF WORK

The overall objective of this study is to understand the mechanism of action of D5 for its use in breast cancer prevention and therapy. Specific aims for the first year (May 2001 - April 2002) include:

Task 1: Perform transformation of normal breast epithelial cells, MCF-12F, using two types of carcinogens, dimethylbenz(a)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU).

Task 2: Evaluate and compare D5 growth effects on normal and transformed cells.

Task 3: Determine whether D5 modulates cell proliferation markers, such as BrdU incorporation into DNA in normal *vs* transformed cells.

Task 4: Determine whether D5 induces apoptosis in transformed MCF-12F cells.

Since MCF-12F are very low in vitamin D receptor (VDR) and estrogen receptor (ER) expression, we used SR^+ breast cancer cells (BT-474 and MCF-7), to perform preliminary experiments that determine possible interaction of D5 with estrogen signaling.

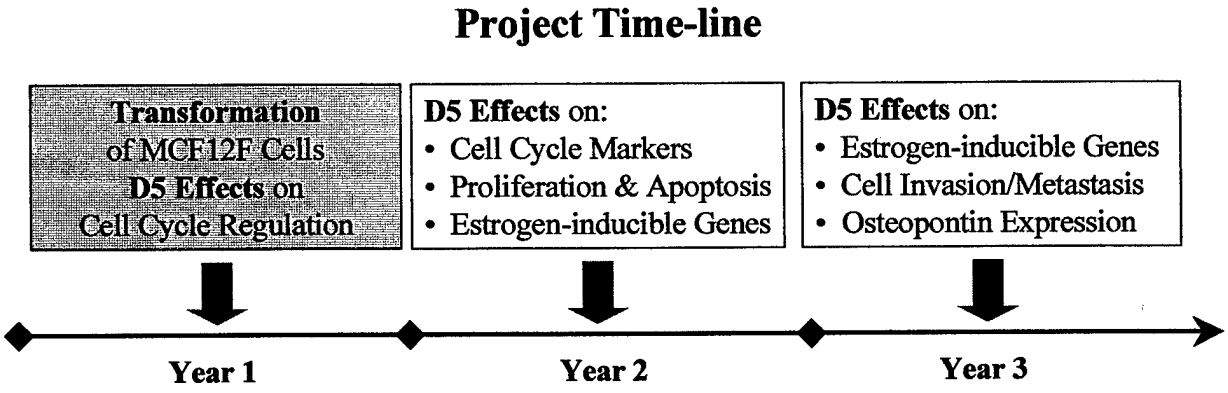

Figure 1: Project time-line for the entire study.

STUDY DESIGN AND METHODS

We have been maintaining the MCF-12F cells according to the ATCC instructions in DMEM: Ham's F12 mixture (1:1) with added insulin, epidermal growth factor, cholera toxin, and hydrocortisone. This cell line had been established with long-term culture in low Ca^{++} media. Therefore, we use 5% chelex-treated horse serum in culture medium. We performed transformation of MCF-12F cells with two different carcinogens, DMBA that needs to be metabolized, and MNU, which is a direct acting carcinogen. Briefly, MCF-12F cells were grown to subconfluency in a tissue culture dish. Cells were incubated with DMBA (2 μg/mL) for 24 hours followed by another 24 hour incubation with fresh DMBA in the media, which caused extensive cell death. Surviving cells were washed with PBS and allowed to grow in fresh media without DMBA. For MNU-induced transformation, the carcinogen was dissolved in acidified saline (pH 5.3) and used within 20 minutes of preparation. Cells were exposed

to MNU (25 μg/mL) twice daily for two days and later allowed to remain in fresh media. Treated cells underwent extensive cell death in serum starved media and the surviving cells were then plated in fresh media. The cell transformation efficiency was tested by soft agar colony formation and tumor incidence in athymic mice. The resulting cell lines were designated MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$.

The growth rate was compared between the normal and transformed cell lines using Coulter cell counter and MTT absorbance assay. Growth effects of D5 treated MCF-12F, MCF-12F$_{DMBA}$, and MCF12F$_{MNU}$ were similarly tested. We also used BrdU incorporation as a marker of cell proliferation. Cells were treated with BrdU for 40 minutes and DNA incorporation of BrdU was determined by immuno-cytochemistry using anti-BrdU (DAKO) and streptavidin peroxidase system. All experiments were performed in duplicates and were repeated at least twice.

Cell cycle analysis was conducted using propidium iodide staining with flow cytometric detection. To detect apoptosis, we used DNA strand break labeling by terminal deoxynucleotidyl transferase (TUNEL) assay. Immuno-histochemistry and RT-PCR were used to determine steroid receptor expression and transcription, respectively, in normal and transformed MCF-12F cells as well as breast cancer BT-474 cells. We are currently studying the expression of cell cycle markers, such as cyclins and cyclin-dependent kinases, and other proteins involved in cell cycle regulation using western blots.

RESULTS

Growth inhibitory effects of D5 on MCF-12F cells were studied using cell count and MTT absorbance assay. No significant difference in cell survival was observed in D5 treated cells as compared to control. The cell count results are summarized in figure 2.

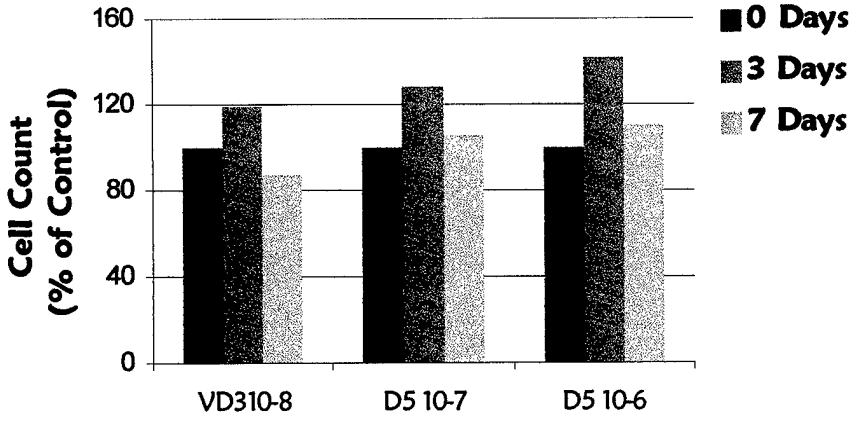

Figure 2. Effect of D5 on MCF-12F Cell Growth

After the transformation of MCF-12F cells into MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$, we characterized and compared its growth rate with that of the parent cell line. The transformed cells are faster growing and have altered morphology. They also showed loss of contact inhibition. The growth rate comparison of the transformed cells with MCF-12F as control is shown in figure 3.

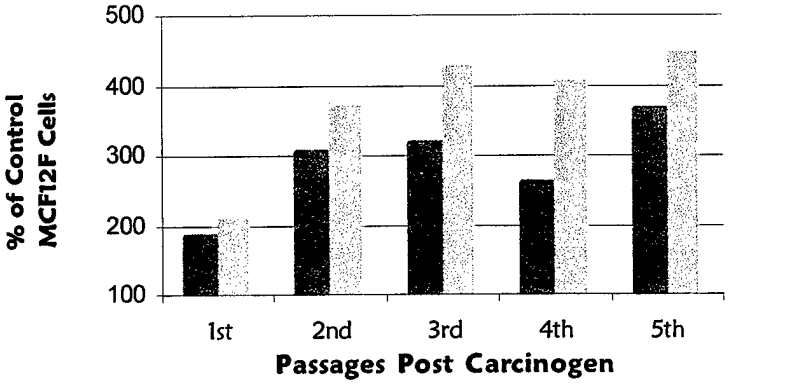

Figure 3. Percent Growth Rate Comparison of Transformed Cells with MCF-12F as Control

Interestingly, the transformed cells showed response to D5 treatment. Figure 4 shows the cell viability with D5 treatment. The growth of transformed cells are significantly reduced by D5 treatment, but no significant effect was observed on MCF-12F cells. It is possible that D5 response may be due to increase in cell cycle progression and decreased doubling time of transformed cells. We are currently performing experiments to determine the effects on D5 on cell cycle related proteins.

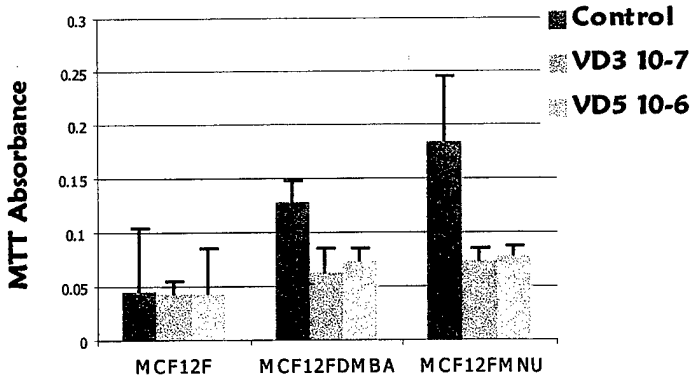

Figure 4. Normal and Transformed MCF12F Cell Viability after 2-day D5 Treatment

We have tested several breast cancer cell lines for D5 response [6] and it appears that D5 mainly inhibits the growth of SR^+ breast cancer cell lines, such as BT-474 and MCF-7. Therefore, we decided to use BT-474 and MCF-7 cells as well to study interaction of D5 with estrogen signalling. Figure 5 shows inhibition of BT-474 cell growth with D5 treatment.

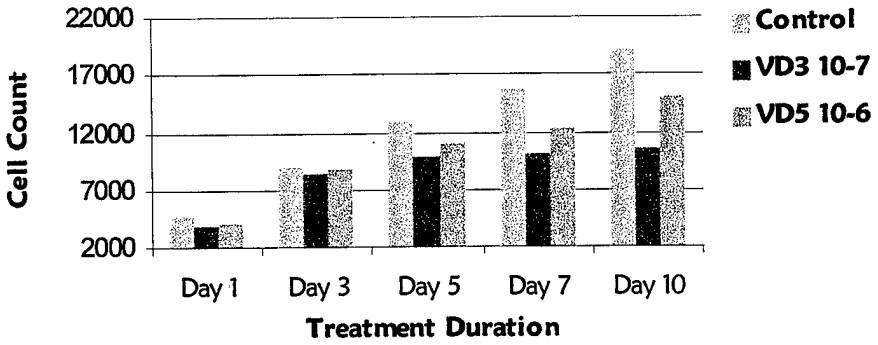

Figure 5. Effect of D5 on BT474 Cell Growth

The cell cycle analysis showed that D5 causes G-1 phase arrest in transformed MCF-12F, MCF-7, and BT-474 cells, but does not significantly alter MCF-12F cell cycle progression. The results are summarized in table I.

Table I. Cell Cycle Analysis by Flow Cytometry.

Cell Line	Treatment	% Cells in G-1 phase
BT-474	Control	60.7
	D5 (1 μM) *	85.3
MCF-7	Control	60.2
	D5 (1 μM) *	71.0
MCF-12F	Control	62.9
	D5 (1 μM)	65.2
MCF-12FDMBA	Control	45.1
	D5 (1 μM) *	65.7
MCF-12FMNU	Control	43.4
	D5 (1 μM) *	59.3

* p value < 0.05

To determine if the cell cycle arrest would be followed by apoptosis we performed the TUNEL assay for DNA strand breaks using Intergen Apoptag® kit. Figure 6 shows the apoptotic brown stained nuclei of transformed MCF-12F and BT-474 cells that were treated with D5.

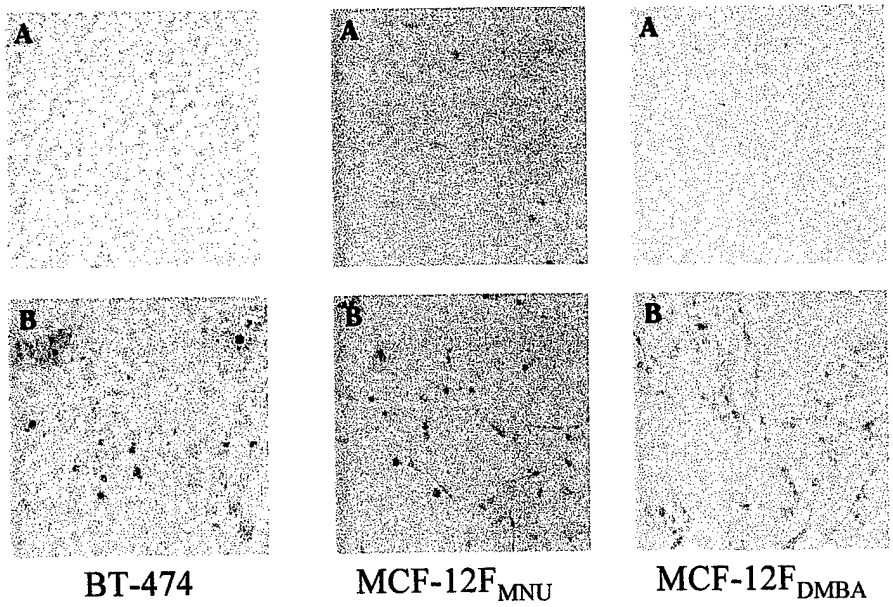

Figure 6. Detection of apoptosis by TUNEL assay in D5 treated transformed MCF-12Fs and BT-474 cells. A is Control and B is D5 (1μM).

To determine the differential gene expression patterns of MCF-12F with MCF-12F$_{MNU}$ cells, we sent out samples for Atlas 8K Human array. The results showed that the transcription of 144 genes was significantly up regulated while149 genes were down regulated in MCF-12F$_{MNU}$ as compared to MCF-12F cells. In comparison of MCF-12F$_{MNU}$ with MCF-12F$_{MNU}$ treated with D5, 95 genes were up regulated and 156 were down regulated. These genes were mostly involved in mitochondrial enzymes as well as cell growth. Interestingly, many genes that have been differentially expressed in MCF-12F$_{MNU}$ were partially restored with D5 treatment. Table II lists a few of these genes.

Similarly, differential expression of D5 treated BT-474 cells was analyzed using Human UniGene 10K arrays. The estrogen inducible genes such as progesterone receptor (PgR) and trefoil factor (pS2) were significantly down regulated with D5 treatment. A list of some important genes altered by D5 in BT-474 cells is given in table III. Based on the results from gene array, we performed immuno-histochemistry and RT-PCR for PgR and ER gene expression and transcription, respectively, in D5 treated BT-474 cells. Figure 7 shows down regulation of PgR expression in BT-474 cells, while figure 8 shows reduced gene transcription of PgR with D5 treatment.

Table II. Micro-array Comparison of MCF-12F with MCF-12F$_{MNU}$ and MCF-12F$_{MNU}$ Control with D5 Treated.

	Differential Expression	
Gene Name	**MCF-12F$_{MNU}$**	**MCF-12F$_{MNU}$ [1α(OH)D5]**
Heat Shock Protein 27 kD	2.7	- 4.0
Prohibitin	4.1	- 2.4
Glutathione Peroxidase 4	- 2.7	3.0
Ornithine Decarboxylase Antizyme 1	- 2.1	2.1
Cystatin B (stefan B)	- 3.4	2.9
Tumor Protein 1 (TCTP1)	- 17.8	16.1
Rho GDP Dissociation Inhibitor α	- 6.5	5.9
BCL2-like 1	- 2.8	2.5
Tissue Inhibitor of Metalloproteinase 1	- 3.1	2.6

Table III. Micro-array Comparison of BT-474 control and D5-treated cells.

Gene Name	**Differential Expression** (fold)	**Statistical Significance**
Estrogen-inducible Genes		
Trefoil Factor 1 (pS2)	5.7 ↓	$p < 0.01$
Trefoil Factor 3 (Intestinal)	3.5 ↓	$p < 0.01$
Progesterone Receptor	3.2 ↓	$p < 0.01$
Vitamin D Regulated Genes		
Vitamin D Receptor	1.1 ↑	NS
Cytochrome P450 (Vitamin D Hydroxylase)	6.3 ↑	$p < 0.01$
Differentiation-related Genes		
Cadherin 18 type 2	3.5 ↑	$p < 0.01$
Matrix Metalloproteinase 9 (type IV Collagenase)	1.5 ↓	$p < 0.05$
Laminin Receptor 1	1.9 ↓	$p < 0.01$
Apoptosis-related Genes		
Caspase 3 (Apoptosis-related Cysteine Protease)	1.7 ↑	$p < 0.01$
Cell Growth Related Genes		
Proliferating Cell Nuclear Antigen	1.2 ↓	NS
Thymidine Kinase 2 (Mitochondrial)	1.9 ↑	$p < 0.01$

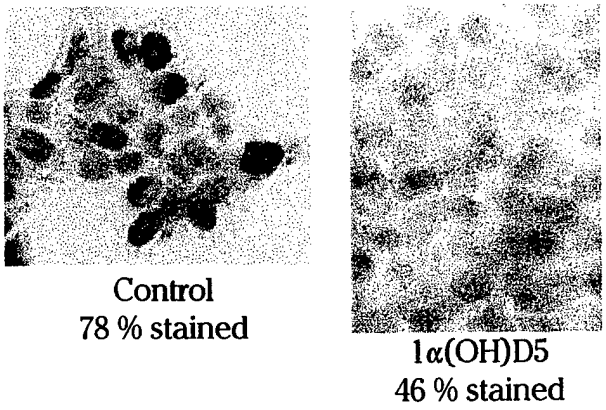

Figure 7. Progesterone receptor expression in BT-474 cells as detected by immuno-histochemistry.

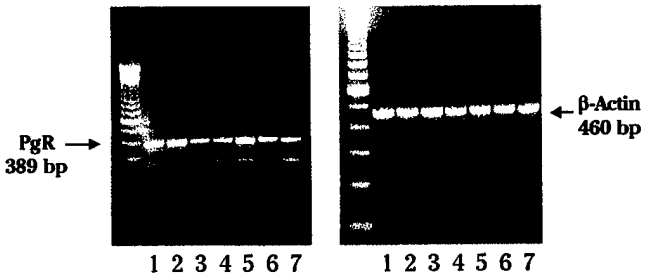

Figure 8. Progesterone receptor transcription in BT-474 cells.

KEY RESEARCH ACCOMPLISHMENTS

- Transformation of non-tumorigenic MCF-12F cells to establish MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$ cell lines.
- Comparison of D5 growth response showing selective growth inhibition of transformed cells by D5.
- Micro-array results showing altered gene transcription in transformed MCF-12F$_{MNU}$ cells.
- Micro-array results indicating that D5 treatment restored transcription of some genes, which were altered by transformation.
- Down regulation of estrogen-inducible genes with D5 treatment in SR$^+$ breast cancer cells (BT-474 and MCF-7).

REPORTABLE OUTCOME

Manuscript: Chemoprevention of Mammary Carcinogenesis by 1α(OH)D5, a Synthetic Analog of Vitamin D. Rajendra G. Mehta, Erum A. Hussain, Rajeshwari R. Mehta, and Tapas K. Das Gupta. *Mutation Research*, 2002 (Accepted for publication).

Abstracts: Vitamin D analog, 1α-hydroxy D5, down regulates estrogen-inducible genes in steroid receptor positive breast cancer cells. Erum A Hussain, Rajeshwari R Mehta, Tapas K Das Gupta, Rajendra G Mehta. AACR Annual Concference, April 2002.

Vitamin D_3 Analog, 1α-hydroxy-24-ethyl Cholecalciferol, Induces Apoptosis and Cell Cycle Arrest in BT-474 Breast Cancer Cells. Erum A Hussain, Rajendra G Mehta. Gordon Cancer Conference, August, 2001.

Presentation: Sigma Xi, 2002 at University of Illinois at Chicago

Cell line development: MCF-12F_{DMBA} and MCF-12F_{MNU}

Funding applied for based on the work supported by this award: Post-doctoral grant applied to Illinois Department of Public Health (IDPH) and received by postdoctoral fellow in Dr Mehta's lab.

CONCLUSIONS AND STUDIES IN PROGRESS

In conclusion our studies indicate a selective action of D5 on transformed cells, but not on normal-like MCF-12F cells. Additionally, our studies indicate down regulation of estrogen inducible genes with D5 treatment. This selective action of D5 combined with weak anti-estrogenic activity can be exploited in both preventive and therapeutic measures for breast cancer[7]. Currently, we are studying cell cycle related proteins and estrogen-inducible genes that are altered by D5 treatment in MCF-12F cells as well as MCF-7 and BT-474 cells.

In the upcoming year, we will determine the interaction of D5 with estrogen signaling in normal and transformed cells by:

a. Transfection of estrogen receptor (ER) into ER^- MCF-12F cells (13-16 months).
b. Comparison of antiproliferative and differentiating effects of D5 in ER^- *vs* ER transfected MCF-12F cells (17-20 months).
c. Assessment of the changes in expression of estrogen inducible genes, such PgR and pS2 with D5 treatment in both normal and transformed MCF-12F cells with or without ER (20-26 months).
d. In addition, we will also perform similar experiments using BT-474 cells.

In the last part of our study we will examine effects of D5 on invasiveness of the transformed and cancer cells:

a. Study D5 effects on cell-extracellular matrix interaction using *in vitro* cell matrigel/collagen migration and outgrowth assays (27-32 months).
b. Study the effects of D5 on expression of vimentin and osteopontin, which are associated with cell migration and metastasis to bone (33-36 months).

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APPENDICES

Chemoprevention of Mammary Carcinogenesis by 1α(OH)D5, a Synthetic Analog of Vitamin D. Rajendra G. Mehta, Erum A. Hussain, Rajeshwari R. Mehta, and Tapas K. Das Gupta. *Mutation Research*, 2002 (Accepted for publication).

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Chemoprevention of Mammary Carcinogenesis by 1α-hydroxyvitamin D5, a Synthetic Analog of Vitamin D3,

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Key words: vitamin D, mammary carcinogenesis, chemoprevention,

Abstract

Numerous analogs of vitamin D have been synthesized in recent years with the hope of generating a compound that retains the anticarcinogenic activity of vitamin D without causing any toxicity. We synthesized such an analog 1α-hydroxy-24-ethylcholecalciferol [1α-hydroxyvitamin D5 or 1α(OH)D5] and showed that it was tolerated by rats and mice at a much higher dose compared to 1α,25 dihydroxy cholecalciferol ($1,25(OH)_2D3$). This property makes it a prime candidate for chemoprevention studies. The chemopreventive efficacy of 1α(OH)D5 was evaluated in the mouse mammary gland organ culture (MMOC) and in the MNU-induced rat mammary carcinogenesis models. In MMOC, 1α(OH)D5 inhibited carcinogen induced development of both mammary alveolar and ductal lesions. *In vivo* carcinogenesis study showed statistically significant reduction of tumor incidence and tumor multiplicity in carcinogen treated rats that were fed 25 to 50 μg 1α(OH)D5/kg diet. There were no adverse effects on plasma calcium concentrations. In order to determine if the effect of 1α(OH)D5 would be selective in suppressing proliferation of transformed cells, its effects on cell growth and proliferation were compared between BT474 (cancer) and MCF12F (non-tumorigenic) human breast epithelial cells. Results showed that 1α(OH)D5 induced apoptosis and cell cycle G1 phase arrest in BT474 breast cancer cells without having any effects on proliferation of the MCF12F cells. As mentioned earlier, in MMOC experiments 1α(OH)D5 inhibited development of carcinogen induced preneoplastic lesions. However, it had no growth inhibitory effects on non-carcinogen treated MMOC. Similarly, non-tumorigenic human breast epithelial cells in explant culture did not respond to 1α(OH)D5, whereas treatment with 1α(OH)D5 induced cell

death in the cancer tissue explants. These results collectively indicate that 1α(OH)D5 at non-toxic concentrations selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. Interestingly, the growth inhibitory effects of 1α(OH)D5 were observed in VDR^+ breast cancer cells, but not in highly metastatic VDR^- breast cancer cells such as, MDA-MB-435 and MDA-MB-231. Hence, it appears that 1α(OH)D5 action may be mediated, in part, by VDR.

Introduction

Conceptually, chemoprevention of cancer can be defined as an intervention in the carcinogenic process by either a naturally derived or a synthetic compound. An agent that blocks, arrests, or reverses the progression of cancer can be termed a chemopreventive agent (1, 2). In practice, this can best be achieved by the dietary administration of chemical agents, which can enhance the physiological processes that protect the organism against the development of malignancy. Current understanding of progression of a normal cell to a transformed cancer cell is summarized in Figure 1. Under experimental conditions, a normal cell could be transformed to an initiated cell in response to carcinogenic or mutagenic stimuli. Although, the initiated cells have potential to develop into malignant cancer, they may or may not form a tumor depending upon the exposure to exogenous and/or endogenous factors. In the absence of growth arrest stimuli, the initiated cell can then advance to a preneoplastic stage leading progressively to malignancy. The chemopreventive agents that suppress the early events in transformation, such as preventing the mutagenic action of chemicals or other factors, are referred to as anti-initiating agents. On the other hand, the chemicals that prevent further progression of the initiated cells into transformed ones are termed as anti-promotional

agents (3, 4). Numerous classes of chemopreventive agents have been reported in the literature, including retinoids, deltanoids, cyclooxygenase inhibitors, inhibitors of polyamine and prostaglandin biosynthesis, lignans, calcium channel blockers, anti oxidants, etc. (5, 6, 7). In this report we have summarized the chemopreventive properties of a newly evaluated vitamin D analog, 1-α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D5].

It has been well established that the active metabolite of vitamin D, 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3] is a steroid hormone and it exhibits potent cell differentiating properties in leukemia cells as well as other cancer cells of epithelial origin (8, 9). The antiproliferative and differentiation-inducing effects of 1,25$(OH)_2$D3 could be of clinical significance in prevention or treatment of the cancer of several target organs (10). However, one major limitation in its clinical application is the fact that the efficacious concentrations of 1,25$(OH)_2$D3 are cytotoxic (11). The effective growth inhibitory concentration of 1,25$(OH)_2$D3 induces dangerously high levels of serum calcium resulting in loss of body weight and soft tissue calcification, which could be lethal (12). This has resulted in generation of several non-toxic but antiproliferative synthetic analogs of vitamin D molecule for the prevention and treatment of cancer. Some of these analogs have been successfully evaluated in their ability to suppress cancer cell growth in culture as well as *in vivo* models (13).

Typically, the structure of vitamin D is divided into four parts (Figure 2): ring A, open ring B, ring CD, and the side chain. The modifications can be made at all four sites, but the alteration of the ring CD is not common due to the rigid structure. The maximum alterations have been made at the open side chain. Nearly 800 analogs of vitamin D have

been synthesized so far and about 300 of them have been evaluated in *in vitro* and *in vivo* experimental models (14, 15). Historically, a comparison of toxicological profile of vitamin D series of compounds, including vitamins D2, D3, D4, D5 and D6, had suggested that vitamin D5 was the least toxic of the D-series of compounds (16). In order to generate an effective but non-calcemic and non-toxic vitamin D analog, we synthesized 1α(OH)D5 (17). The structure of 1α(OH)D5 is shown in Figure 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic pathway involves its association with high affinity specific vitamin D receptor (VDR) that belongs to steroid receptor superfamily of ligand activated transcription factors (18, 19, 20). This is consistent with the well-known mode of action of the steroid hormones. The VDR has been identified in a variety of tissues such as breast, prostate, liver, fibroblasts, colon, and lungs (21) in addition to the previously known target organs that included intestine, kidney, and bone. The VDR mRNA is about 4.6 kb that translates to a 50 kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell yielding 10 to 100 femtomoles per mg of total protein. In order for VDR to function, it needs to bind specific DNA sequences and interact with vitamin D response elements (VDRE) (22). The natural metabolite $1,25(OH)_2D3$ transactivates VDRE in VDR^+ cells but fails to show interaction in VDR^- cells. Hence, vitamin D analogs that are able to transactivate VDR-VDRE are mainly mediating their action via genomic pathways. Non-genomic vitamin D actions have been studied mostly in relation to calcium and phosphorus metabolism, and to a lesser extent with respect to chemoprevention. The rapid responses involve a putative membrane receptor of vitamin D that signals to modulate calcium channel activity in a cell. This may lead to exocytosis

of calcium bearing vesicles from lysosomes. The non-genomic pathway for vitamin D action has been extensively reviewed elsewhere (23, 24). For this article, we have listed the chemopreventive properties and possible mode of action of 1α(OH)D5.

Materials and Methods:

Cell lines:

Non-tumorigenic estrogen receptor negative (ER^-), progesterone receptor negative (PgR^-) and low VDR breast epithelial cell line MCF12F, ER^+ PgR^+ VDR^+ breast cancer cell lines, BT474 and MCF7, and ER^- PR^- VDR^- MDA-MB-231 and MDA-MB-435 cell lines were purchased from American Type Culture Collection (ATCC), Bethesda, MD and maintained in our laboratory according to the ATCC recommendations.

Mouse Mammary Gland Organ Culture (MMOC):

The detailed procedure for culturing mammary glands from Balb/c mice have been previously reported in the literature (17, 25) and outlined in Figure 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Waymouth's 752/1 medium under 95% O_2 and 5% CO_2 at 37°C. The glands respond to growth promoting hormones insulin, prolactin, aldosterone and hydrocortisone, and differentiate into distinct alveolar structures. Exposure of glands to 7,12 dimethylbenz(a)anthracene (DMBA) for 24 hours on day 3 of culture results in the development of precancerous mammary alveolar lesions (MAL). If the growth-promoting medium contains estrogen and progesterone instead of aldosterone and hydrocortisone, the glands develop mammary ductal lesions (MDL) with DMBA treatment (26). We performed a dose response study to compare the effects of 1α(OH)D5 with control on MAL and MDL. Additionally we determined the effects of 1α(OH)D5 on normal

mammary glands, where the glands were incubated with growth promoting hormones and 1μM 1α(OH)D5 for 6 days without DMBA treatment. The glands from these MMOC experiments were fixed, stained and analyzed for morphological characteristics and cell growth.

Cell Cycle Analysis by Flow Cytometry:

We used flow cytometric analysis for determination of cell cycle as described by Vindeløv *et al* (27). Breast epithelial non-tumorigenic and cancer cells were detached by trypsinization and were harvested. The cells were washed twice with PBS and pelleted. The pellet was resuspended and fixed in 85% ice-cold ethanol. After fixing, the cells were centrifuged and resuspended in citrate buffer and then incubated with NP-40, trypsin, and spermine for 15 minutes. This was followed by incubation with trypsin inhibitor and RNAse A. The cells were then stained with 0.04% propidium iodide solution. Approximately 10,000 cells were analyzed for DNA content using Beckman-Coulter EPICS Elite ESP flow cytometer. Multicycle analysis software was used to determine the percentage of cells in various stages of cell cycle.

Apoptosis:

Programmed cell death was evaluated using acridine orange staining. Briefly, 50 μl suspension of breast epithelial cells was stained with 2 μl of acridine orange/ethidium bromide solution (100 μg/mL acridine orange and 100 μg/mL ethidium bromide in PBS). Cells were layered on a glass slide and examined under a fluorescent microscope with a 40x objective lens using a flourescein filter. For comparison of number of cells undergoing apoptosis, about 100 cells were counted on each slide.

Mammary carcinogenesis:

The procedure for induction of mammary cancers by N-methyl-N-nitrosourea (MNU) in Sprague/Dawley female rats has been described in detail previously (28) and illustrated in Figure 4. Briefly, 100 days old female Sprague/Dawley rats were injected subcutaneously with 50 mg/kg MNU prepared in acidified saline. Animals received either placebo or 1α(OH)D5 supplemented as 25 or 50 μg/kg diet. Animals were sacrificed after 230 days of treatment. Mammary tumors were identified by palpation as well as necroscopy. Results were reported as effects of 1α(OH)D5 on the incidence, multiplicity, and latency of tumor development and data were subjected to appropriate statistical analyses.

Effects of 1α(OHD5 on normal and malignant breast tissue:

Breast tissues were obtained from women undergoing mastectomy or lumpectomy. Explants were maintained in MEME medium, containing 5% stripped fetal bovine serum. The effects of 1μM 1α(OH)D5 were determined on these tissues by evaluating cell morphology, apoptosis, and expression of Ki 67. Effects of 1α(OH)D5 on the cell morphology and Ki67 were compared between the normal and adjacent cancer tissue from the same patient.

Results and Discussion:

Synthesis and Toxicity of 1α(OH)D5:

Nearly 300 analogs of $1,25(OH)_2D3$ have been evaluated in various experimental systems in the hope of generating more efficacious analogs with reduced toxicity. Among the analogs evaluated, only a few have shown potent chemopreventive and therapeutic activity. These analogs, which include EB1089 (29), KH1060 (30), R024-5531 (31), 22-Oxacalcitriol (32) are relatively nontoxic at effective concentrations in experimental

models. The hexafluoro analog of 1,25$(OH)_2$D3, R024-5531, has no calcemic activity, while other analogs do express dose related calcemia (33, 34). Since it had been reported previously that vitamin D5 is the least toxic series of vitamin D compounds, we synthesized 1α(OH)D5 with the intention of testing its chemopreventive potential. The chemical synthesis of 1α(OH)D5 has been previously reported from our laboratory (17).

Since calcemic activity is an obstacle to the development of effective vitamin D analogs suitable for clinical use, we determined serum calcium and phosphorous concentrations after treating vitamin D deficient rats with 1,25$(OH)_2$D3 and 1α(OH)D5. As mentioned earlier, male Sprague-Dawley rats (8-10 per group) were fed vitamin D deficient diet for 3 weeks and baseline serum calcium levels were determined. Rats showing ≤ 6 mg/dL serum calcium were given 1α(OH)D5 for 14 days. Subsequently, serum calcium concentrations were measured. Results showed that 1,25$(OH)_2$D3 significantly ($p < 0.001$) increased serum calcium concentration at a daily dose of 0.042 μg/kg diet, whereas there was no elevation in serum calcium levels among 1α(OH)D5 treated animals (17). A similar experiment was carried out using vitamin D sufficient regular diet. Female Sprague-Dawley rats were treated with various concentrations of 1,25 $(OH)_2$D3 (0.8 to 12.8 μg/kg diet) and 1α(OH)D5 (6.4 to 50 μg/kg diet) for two months. Calcium concentration was increased by 1,25 $(OH)_2$D3 treatment, while no serum calcium elevation was observed in 1α(OH)D5 treated (25 μg/kg diet) animals (Table 1). There was no effect on the final body weight at any dose of 1α(OH)D5 used in this study. These results indicate that 1α(OH)D5 is considerably less toxic compared to the natural hormone. More recently, we completed an extensive preclinical toxicity study in both sexes of rats and dogs under Good Laboratory Practice (GLP). Results showed

that dogs are relatively more sensitive to the higher dose of 1α(OH)D5 as compared to rats. We concluded from those studies that 1α(OH)D5 is mildly calcemic in dogs at concentrations higher than 10 μg/kg diet. The non-calcemic analog R024-5531 shows toxicity in rats without having an effect on serum calcium concentrations. On the other hand, 1α(OH)D5 can be tolerated at a higher concentration without other toxicity outcomes.

Chemoprevention of Mammary Carcinogenesis by 1α(OH)D5:

Chemopreventive properties of 1α(OH)D5 have been evaluated in two experimental systems in our laboratory. These include MMOC and MNU-induced mammary carcinogenesis in Sprague-Dawley rats. Mouse mammary glands respond to DMBA and develop preneoplastic mammary alveolar as well as ductal lesions in organ culture. As shown in Figure 3, efficacy of a potential chemopreventive agent can be assessed in this assay. If the agent is present and effective prior to carcinogen treatment its effects are considered as anti-initiation, whereas, if it is effective subsequent to carcinogen then its effect are anti-promotional. Both types of effects can be determined using the MMOC model. We showed previously that 1α(OH)D5 inhibits the development of mammary lesions in a dose responsive manner (17). However, it requires 10 fold higher concentration compared to the effective concentration of $1,25(OH)_2D3$. The most effective dose of $1,25(OH)_2D3$ in suppressing > 60% incidence of MAL is 10^{-7}M, while 1α(OH)D5 is equally effective at 10^{-6}M without showing cytotoxicity. We also evaluated 1α(OH)D5 effects in MDL model (25). The results are summarized in Figure 5. We found 1α(OH)D5 to be equally effective both against alveolar and ductal lesions.

Since most of the effects of vitamin D are mediated through VDR, we determined VDR induction by 1α(OH)D5 in MMOC as well as in breast cancer cell lines (17). There was a significant increase in the expression of VDR in the epithelial cells of MMOC as determined by immunocytochemistry. Additionally, 1α(OH)D5 also upregulated the expression of TGFβ in the epithelial cells of MMOC (15).

Based on these results, it was reasonable to expect chemopreventive activity of 1α(OH)D5 in an *in vivo* model. Prior to conducting *in vivo* carcinogenesis studies, a dose tolerance study was conducted for Sprague-Dawley rats. Animals were provided with increasing concentrations of 1α(OH)D5, ranging from 1 to 100 μg/kg diet for six weeks. The animals did not show any adverse effects at any concentration of 1α(OH)D5 while the natural hormone was toxic at 3.5 μg/kg diet. For the MNU-induced mammary carcinogenesis studies, animals were fed 1α(OH)D5 at 25 and 50 μg/kg diet for three months. The experimental diet was given to the animals one week prior to the carcinogen treatment and continued till the end of the study. Results are shown in Table 2. The results indicated a dose dependent suppression of tumor incidence by 1α(OH)D5. This was accompanied by reduction in tumor multiplicity and increase in tumor latency (28). These results are comparable with those of EB1089, R024-5531, and KH1060. The *in vivo* results as well as the results from MMOC clearly suggest a potential for 1α(OH)D5 to be developed as chemopreventive and therapeutic agent.

Selectivity of 1α(OH)D5 action for transformed cells:

We compared the growth effects of 1α(OH)D5 in various steroid receptor positive as well as negative breast cancer cell lines. These cell lines included non-tumorigenic MCF12F breast epithelial cells, ER^+ PgR^+ VDR^+ BT474 and MCF7, and ER^- PgR^- VDR^-

highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both 1,25 $(OH)_2D3$ and 1α(OH)D5 were efficacious in suppressing cell proliferation of ER^+, PR^+ and VDR^+ BT474, T47D, ZR75 and MCF7 breast cancer cells. These compounds induced differentiation of ER^- PgR^- VDR^+ BCA-4 cells (35), but did not show any growth effects in MDA-MB-435 and MDA-MB-231 cells. Other researchers have also reported similar results with other vitamin D analogs (36). Although our results indicate that presence of VDR is necessary to potentiate vitamin D affect, it does not explain lack of vitamin D affect on MCF12F that express low levels of VDR. In order to examine whether 1α(OH)D5 inhibits cell proliferation in transformed cells only, we evaluated effects of 1α(OH)D5 on non-tumorigenic breast epithelial cells and compared it to the effects on BT474 breast cancer cells. As shown in Figure 6, incubation of MCF12F breast epithelial cells for 6 days with 1α(OH)D5 at 1μM concentration did not result in suppression of cell proliferation as determined by MTT absorbance assay. On the other hand, there was a significant inhibition of proliferation in both MCF7 and BT474 cells with 1α(OH)D5 treatment. These results suggested that the effect of vitamin D analog might be selective for transformed cells.

To confirm the selectivity of 1α(OH)D5 for transformed breast cancer cells, we conducted three separate experiments. In the first experiment, we compared efficacy of 1α(OH)D5 between MCF12F cells with that of MNU-transformed MCF12F ($MCF12F_{MNU}$) cells. The $MCF12F_{MNU}$ cells have recently been established in our laboratory (unpublished data). The $MCF12F_{MNU}$ cells have altered morphology and growth properties as well as different growth factor requirements (Hussain and Mehta, unpublished data). Incubation of MCF12F and $MCF12F_{MNU}$ with 1μM 1α(OH)D5 for 6

days resulted in 50% growth inhibition in $MCF12F_{MNU}$ cells without having any significant effects on MCF12F growth.

In a second study, effects of 1α(OH)D5 were determined in mammary glands using the MMOC model. Mammary glands respond to growth promoting hormones and develop structurally differentiated alveoli within 6 days in culture. Incubation of glands with 1μM 1α(OH)D5 for 6 days did not affect the growth promoting effects of insulin, prolactin, aldosterone, hydrocortisone, estrogen and progesterone (Figure 7). Contrarily, 1α(OH)D5 showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Figure 5).

Experiments to determine selectivity of 1α(OH)D5 action against transformed cells were further extended to human tissues. Effects of 1α(OH)D5 on the explants derived from normal breast tissues were compared with that of cancer tissue. Breast tissue samples were obtained from women undergoing mastectomy or lumpectomy at the University of Illinois at Chicago Hospital. Tissue explants of tumors and normal adjacent cells were incubated for 72 hours in the MEME containing 5% fetal calf serum with or without 1α(OH)D5 at 1μM concentration. Tissue sections were histopathologically evaluated and Ki67 expression was determined. Results showed that the histopathology of control and 1α(OH)D5 treated normal breast tissue was identical with no difference in apoptosis and Ki67 expression. On the other hand, the histological sections of the cancer tissue explants showed extensive apoptosis within the tissue with condensed chromatin and reduced Ki67 expression after 72-hour incubation with 1α(OH)D5 (Mehta, unpublished data). Taken together, these results indicate that in human breast epithelial

tissues, 1α(OH)D5 is selective for its effects on transformed or pre-cancerous cells but shows no effect normal breast epithelial cell growth.

Mechanism of 1α(OH)D5 action:

Effects of 1α(OH)D5 have also been evaluated in several breast cancer cell lines. Although, these studies do not focus directly on chemoprevention, they do provide excellent insight into mechanism of action of 1α(OH)D5 and its efficacy as an anti-proliferative agent. We had reported that in ER^+ PgR^+ breast cancer cells, 1α(OH)D5 inhibited cell growth by inducing apoptosis as well as differentiation, whereas in ER^- but VDR^+ cells, it induced cell differentiation without the induction of apoptosis (35). Similar results have also been reported by numerous investigators using other analogs of vitamin D (37). The data from these studies consistently reported that breast cancer cells expressing VDR respond to vitamin D analogs. These results suggested that the mode of action of 1α(OH)D5 was dependent not only on expression of VDR but also on the expression of ER and ER-inducible genes such as PgR.

The effects of 1α(OH)D5 on cell cycle were determined using breast cancer cells. The BT474 cells were treated with 1μM 1α(OH)D5 for various time points and processed for FACS analysis as described in the methods section. Results showed that 70% of the control cells were distributed in G1 phase, whereas treatment with 1α(OH)D5 induced growth arrest with 84% cells in G1 phase of the cycle. The results are summarized in Table 3. In agreement with our cell proliferation data, there was no difference between the distribution of cells in various cell cycle stages for MCF12F and MBA-MD-231 cells with 1α(OH)D5 treatment. Both MDA-MB-231 and MDA-MB-435 cells are devoid of steroid receptors and therefore, these cells were not expected to

respond to 1α(OH)D5 treatment. These results further confirm that the action of 1α(OH)D5 may, in part, be mediated by VDR.

The mechanism of action of 1α(O)D5 was further evaluated by determining the ability of the cells to undergo apoptosis. The BT474 cells were treated with $1,25(OH)_2D3$ or 1α(OH)D5 for 72 hours and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Figure 8 shows that BT474 cells underwent apoptosis with 1α(OH)D5 treatment as determined by acridine orange and ethidium bromide staining. The stain distinguishes live cells with those that are undergoing apoptosis. On the other hand, no apoptosis was observed in ER^-PgR^- VDR^+ BCA-4 cells, but there was an induction of differentiation as shown by casein, lipids and α2integrin expression (35).

The chemopreventive agents are being developed mostly for people with no disease but at high risk of developing cancer. Here we showed that the vitamin D analog might be selective for transformed cells. The population at a high risk of developing cancer is assumed to be initiated for carcinogenesis and as we have shown, the initiated cells respond well to 1α(OH)D5. In addition we also showed here that 1α(OH)D5 is effective against steroid responsive cancer cells. Can this be considered as a possible chemopreventive and therapeutic agent? Moreover, can it be given in combination with other agents? These are some of the questions we endeavor to answer in our future correspondence.

It is unclear as to where chemoprevention ends and chemotherapy begins. However, the clear principle and prerequisite of chemoprevention is that the agent should

not have any adverse effects. The lack of toxicity of 1α(OH)D5 may provide a rationale for its role in chemoprevention and therapy.

In summary, we have described here chemopreventive properties of a relatively new non-toxic analog of vitamin D, 1α(OH)D5, against mammary carcinogenesis models. In addition, our results suggest that 1α(OH)D5 may be selectively active against the transformed cells without showing adverse effects on normal breast epithelial cells.

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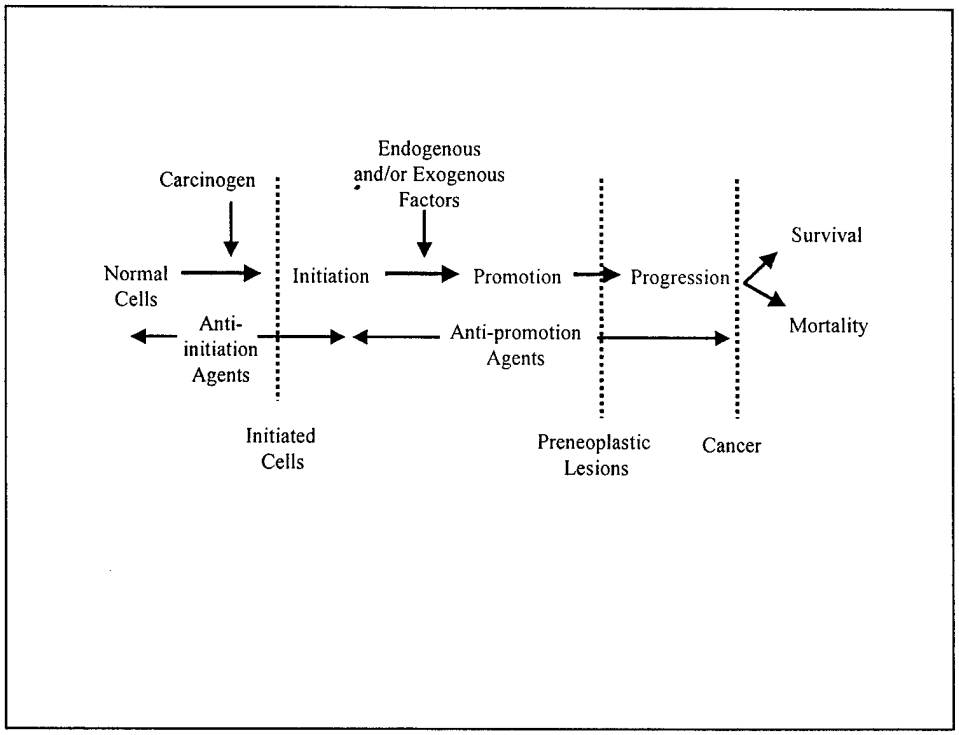

Figure 1. Schematic diagram to show stages in mammary carcinogenesis and potential points of intervention by chemopreventive agents.

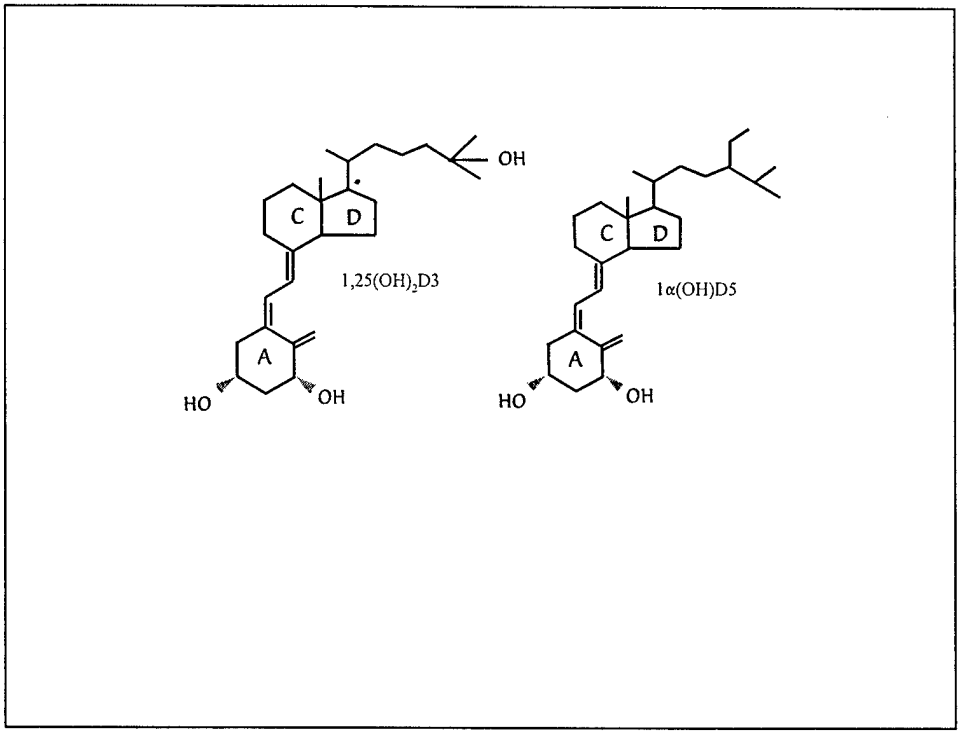

Figure 2. Structural representation of $1,25(OH)_2D3$ and its analog $1\alpha(OH)D5$.

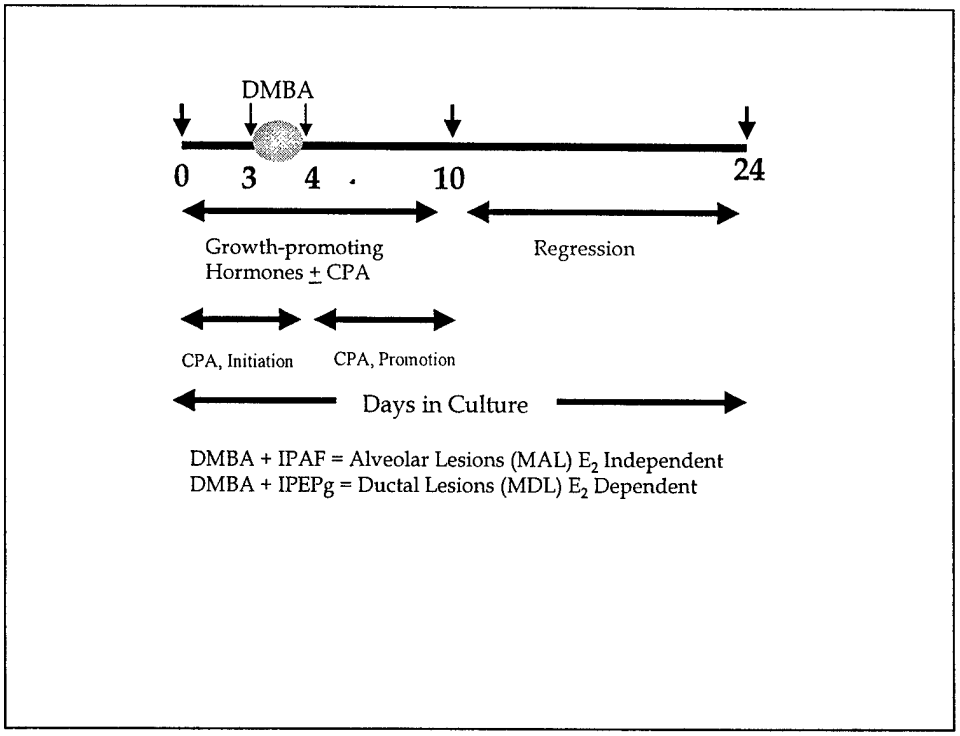

Figure 3. Experimental design for chemoprevention in mouse mammary gland organ culture (MMOC). DMBA: 7,12-dimethylbenz(a)anthracene, CPA: chemopreventive agent, IPAF: insulin + prolactin + aldosterone + hydrocortisone, IPEPg: insulin + prolactin + estradiol + progesterone, MAL: mammary alveolar lesions, MDL: mammary ductal lesions.

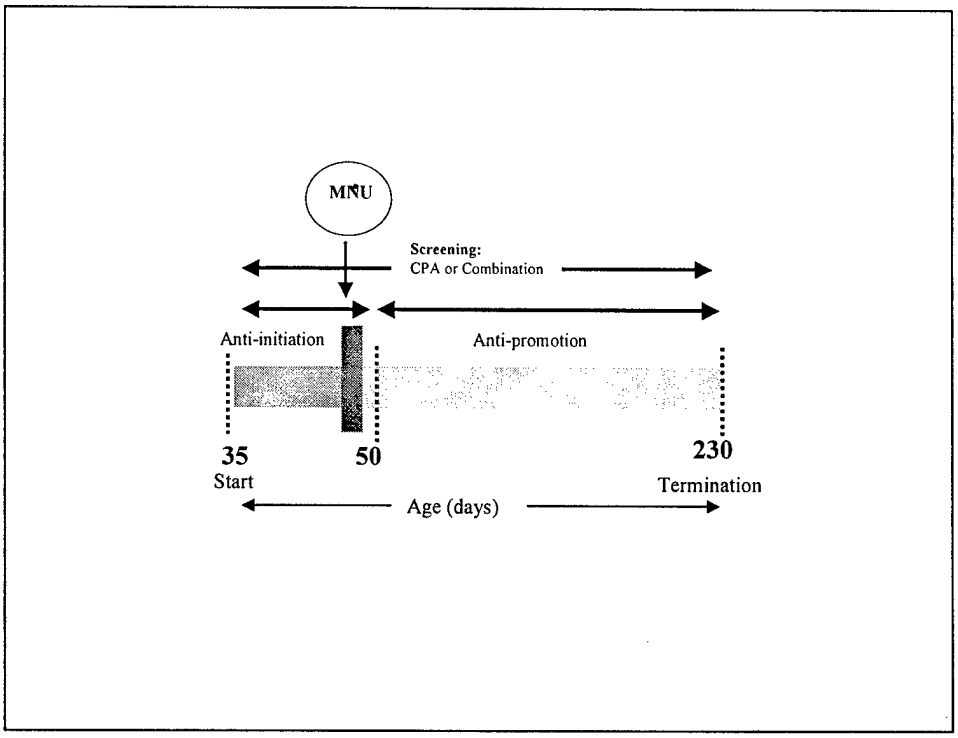

Figure 4. Schematic diagram to show *in vivo* model of chemoprevention in N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague-Dawley rats. CPA: chemopreventive agent.

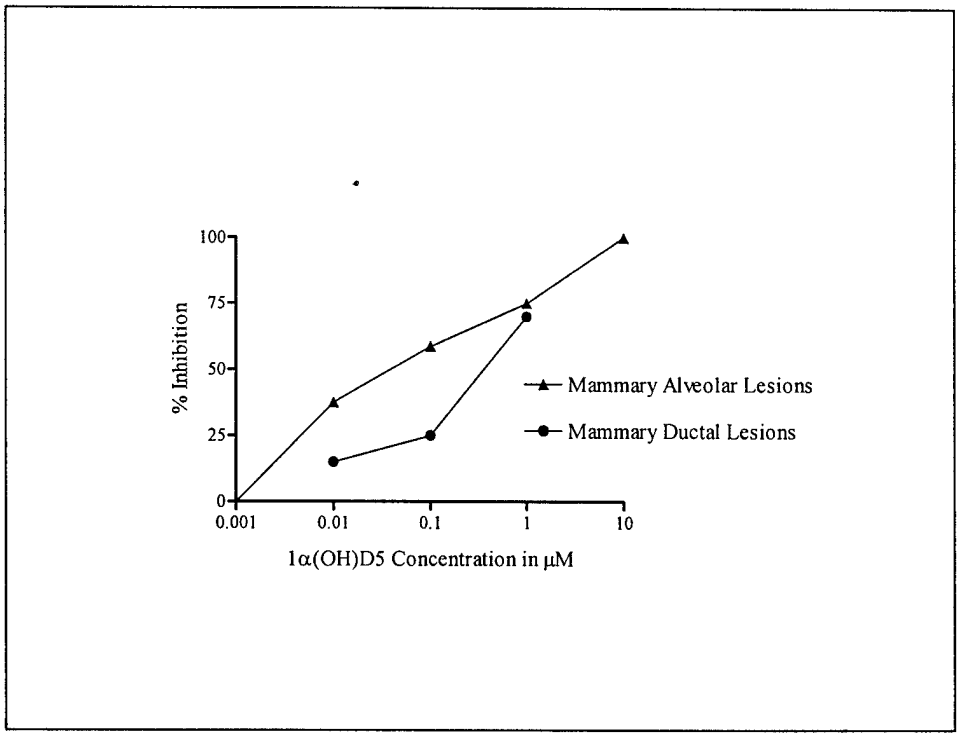

Figure 5. Effect of 1α(OH)D5 on mouse mammary organ culture (MMOC). The glands were incubated with 1 μM 1α(OH)D5 for 10 days. The glands were fixed and evaluated for inhibition of preneoplastic lesions in relation to control. Data shows % inhibition of preneoplastic MAL and MDL with $1\alpha(OH)D_5$ treatment. MAL: mammary alveolar lesions, MDL: mammary ductal lesions.

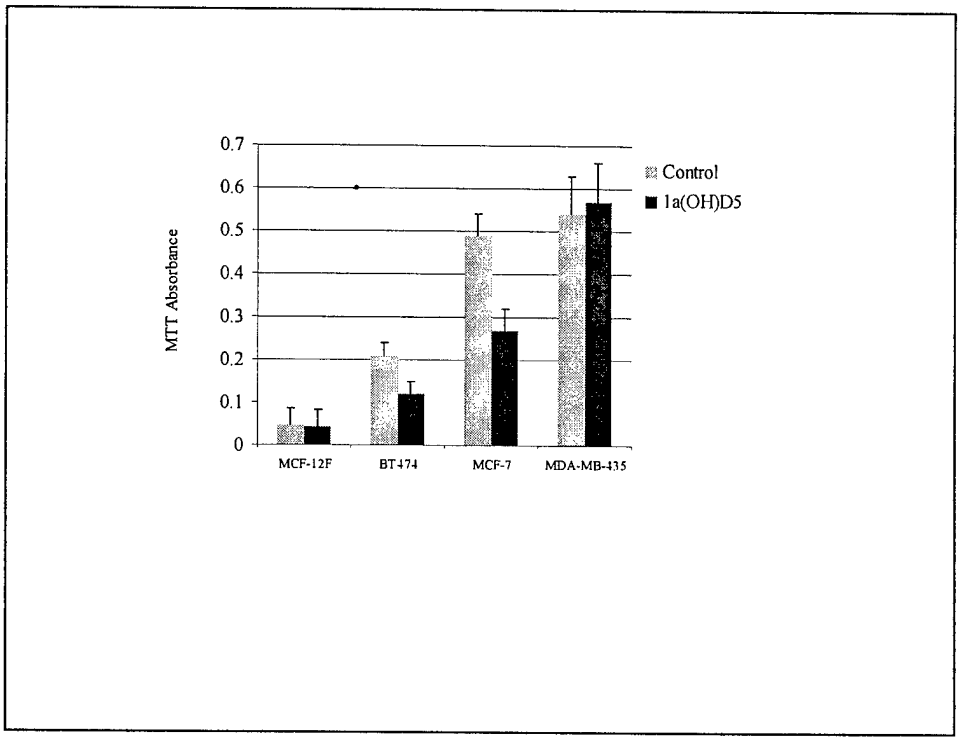

Figure 6. Effects of 1α(OH)D5 on viability of non-tumorigenic and cancer breast epithelial cells. Different cell lines were treated with 1 μM 1α(OH)D5 for 2 days and incubated with MTT for 2 hours. The cells were lysed and washed prior to reading absorbance at 550 nm. MTT absorbance is proportional to the number of live cells.

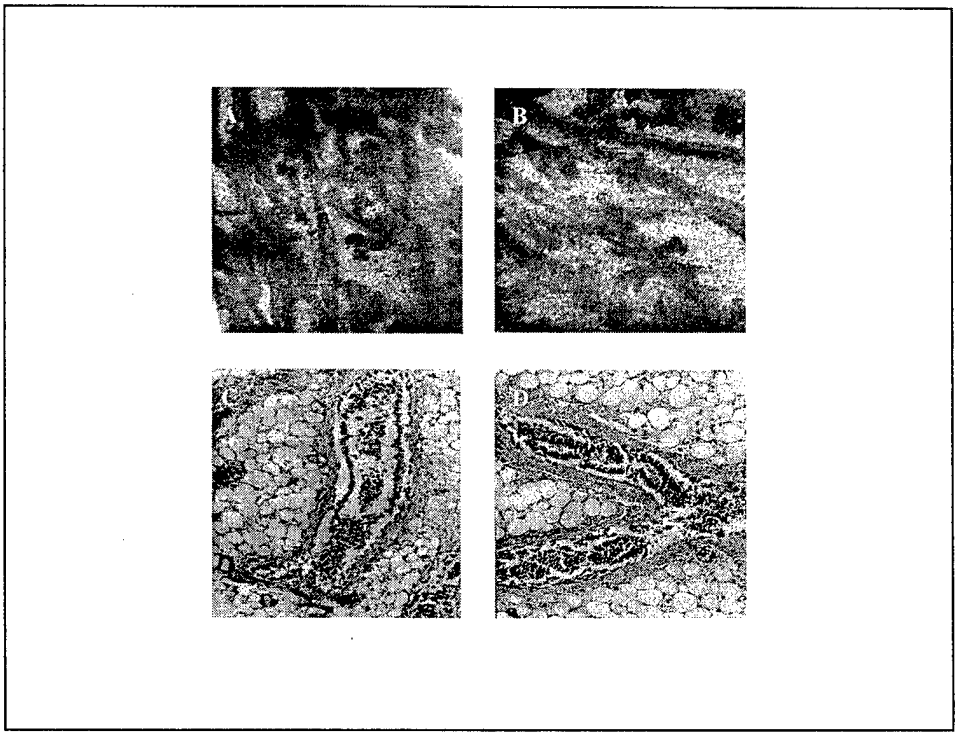

Figure 7. The 6-day mouse mammary organ culture (MMOC) was performed without the carcinogen treatment. The data shows similar growth in both the control and 1α(OH)D5 treated glands. A: control & B: 1α(OH)D5, fixed and stained with carmine. C: control & D: 1α(OH)D5, fixed, sectioned, and stained with *H & E*.

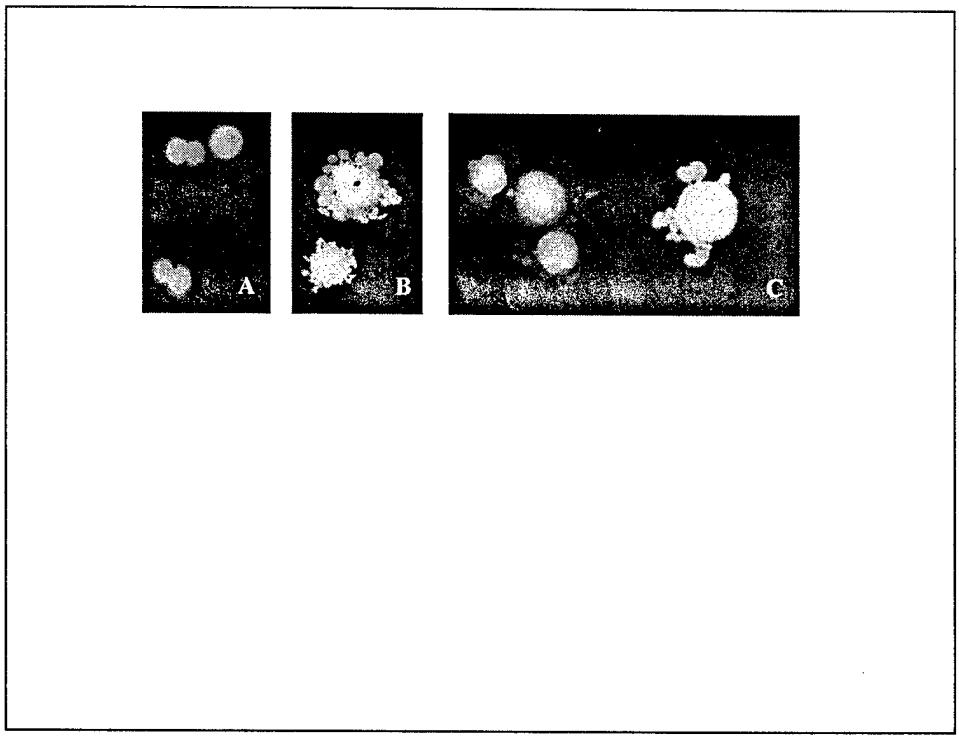

Figure 8. Induction of apoptosis in BT474 cells by 1 μM 1α(OH)D5, as determined by acridine orange and ethidium bromide staining. A: control, B: $1,25(OH)_2D3$ (0.1 μM) , C: 1α(OH)D5 (1 μM).

Table 1. Effects of 1α(OH)D5 treatment on serum calcium and phosphorous levels in Sprague-Dawley rats

Agent	Dose (μg/kg)	Serum Ca (mg/dL)	Serum P (mg/dL)	BW (% gain)
None		6.3	3.6	100
$1,25(OH)_2D3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5	8.9	70
1α(OH)D5	6.4	6.3	7.2	99
	12.5	6.2	7.2	97
	25.0	6.5	7.1	98
	50.0	ND	ND	113

Table 2. Chemoprevention of MNU-induced mammary carcinogenesis by 1a(OH)D5 in rats

Treatment	Dose (μg/kg)	N	Incidence (%)	Multiplicity	Final BW (g)
Control	0	15	80	1.6	228
1α(OH)D5	25	15	53	1.2	230
1α(OH)D5	50	15	47	0.8	226

Table3. Effects of 1α(OH)D5 on cell cycle phases in breast epithelial cell lines

		% G-1	% S	% G-2	G-1/G-2
BT474	Control	60.7	30.5	8.8	6.9
	$1,25(OH)_2D3$	71.6	22.1	6.3	11.4
	1α(OH)D5	85.7	10.3	4.0	21.4
MCF-7	Control	61.2	28.6	10.1	6.1
	$1,25(OH)_2D3$	71.9	19.3	8.8	8.2
	1α(OH)D5	70.0	20.4	9.6	7.3
MDAMB435	Control	22.8	31.3	45.9	0.5
	$1,25(OH)_2D3$	21.1	33.0	45.3	0.5
	1α(OH)D5	21.1	23.6	55.3	0.4
MCF-12F	Control	72.4	16.2	11.4	6.4
	$1,25(OH)_2D3$	61.1	20.2	19.0	3.2
	1α(OH)D5	67.3	16.2	16.5	4.1

Vitamin D_3 Analog, 1α-Hydroxy-24-Ethyl Cholecalciferol, Induces Apoptosis and Cell Cycle Arrest in BT-474 Breast Cancer Cells.

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Breast cancer is the second major cause of cancer deaths among women in the US. Several epidemiological studies have shown an increased risk of breast cancer mortality with lower serum vitamin D_3 (VD_3) levels. It is well established that VD_3 induces cell differentiation and inhibits cellular proliferation *in vitro* at concentrations which would be hypercalcemic *in vivo*. Therefore, various non-calcemic analogs of VD_3 have been synthesized and characterized. Previously, we had reported efficacy of a non-calcemic VD_3 analog, 1α-hydroxy-24-ethyl Cholecalciferol (VD_5), in inhibiting growth of MCF-7 breast cancer cells and preventing development of DMBA-induced preneoplastic lesions in mouse mammary organ culture. In addition, VD_5 had been shown to reduce tumor incidence and multiplicity in carcinogen-treated rats. To understand the mechanism of action of VD_5, we used steroid hormone receptor positive BT-474, and steroid hormone non-responsive MDA-MB-231 and MDA-MB-435 breast cancer cell lines as well as non-tumorigenic MCF-12F cell line. VD_5 treatment resulted in significant cell growth inhibition in BT-474 cells (58.9 % of control). No significant growth inhibition was seen in MCF-12F, MDA-MB-435, or MDA-MB-231 cells. Acridine Orange/Ethidium Bromide staining showed marked apoptosis in BT-474 cells while MDA-MB-435 and MCF12F showed no difference from control. Cell cycle analysis confirmed the growth inhibition induced by VD_5 in BT-474 cells; G-1 phase was 141.2% of control while S phase was reduced by 66.2%. Immunohistochemical analysis showed down regulation of progesterone receptor with VD_3 and VD_5 treatment in BT474 cells with no effect on the expression of vitamin D receptor (VDR) or estrogen receptor. Since BT-474 cells have high expression of VDR, we conclude that growth inhibitory effects of VD_5 are mainly mediated through VDR. VDR is not expressed in normal breast tissue but it is expressed in breast tissue during early cancer progression. Therefore, VD_5 may serve as an effective chemopreventive agent. Further studies on the mechanism of action of VD_5 are warranted to explore its potential use in breast cancer chemoprevention (Supported by DAMD17-01-1-0272).

Vitamin D analog, 1α-hydroxy D5, down regulates estrogen-inducible genes in steroid receptor positive breast cancer cells.

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Since the early 1930s, epidemiologists have reported an increased risk of breast cancer mortality with lower serum vitamin D levels. These observations led to *in vitro* studies which established that the active metabolite of vitamin D, 1α,25$(OH)_2D_3$ (VD_3), is a potent inducer of cell differentiation and an inhibitor of cellular proliferation. However, VD_3 is efficacious at concentrations that would be hypercalcemic *in vivo*. Therefore, various non-calcemic analogs of VD_3 have been synthesized and characterized. Previously, we had reported that 1α-hydroxy D5 (D5), a non-calcemic analog of VD_3, induced apoptosis in steroid receptor positive breast cancer cells. However, it induced differentiation in estrogen receptor (ER) and progesterone receptor (PR) negative but vitamin D receptor (VDR) positive breast cancer cells. Breast cancer cells which were negative for VDR, ER and PR showed no effect of D5 treatment. Therefore, to understand the mechanism of action of D5, we used ER^+, PR^+, and VDR^+ BT-474 breast cancer cells for the present study. D5 treatment (1 μM) resulted in significant growth inhibition of BT-474 cells (58.9 % of control). Acridine Orange/Ethidium Bromide staining suggested marked apoptosis in these cells upon treatment with D5. Cell cycle analysis revealed that D5 treatment increased cells in G-1 phase by 141.2% and reduced cells in S phase by 66.2%. In addition, dietary intake of D5 (25 μg/kg diet) also suppressed growth of BT474 xenograft in athymic mice. In order to determine whether D5-mediated growth inhibition is mediated by its interaction with ER or ER-inducible genes, differential mRNA expression of D5 treated BT474 cells was analyzed using Uni Gene 1 Micro-arrays (10,000 genes). Data from Uni Gene 1 Micro-arrays showed statistically significant down regulation of transcription of estrogen-inducible PR (3.2 fold) and trefoil factor 1/pS2 (5.7 fold) mRNA in D5 treated BT474 cells. Additionally, D5 treatment up regulated caspase 3 mRNA to 1.7 fold. Based on these data, we performed RT-PCR on D5 treated BT474 cells. RT-PCR results showed that D5 did not affect the mRNA levels of ERβ, but it down regulated ERα as well as PR mRNA. These results were further confirmed by immunohistochemical analysis. The down regulation of PR levels with VD_3 and D5 treatment (52 & 59% of control, respectively) were detected in BT474 cells. These results indicate that in steroid receptor positive breast cancer cells, D5 exerts its growth inhibitory effects partly by down regulating ERα as well as estrogen-inducible genes. (Supported by CA82316, DAMD-17-99-1-9223, and DAMD-17-01-1-0272).