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Award Number: DAMD17-01-1-0272

TITLE: Mechanism of Action of a Novel Analog of Vitamin D3, 1α-hydroxy-24-ethyl Cholecalciferol (D5), in Normal and Transformed Human Breast Epithelial Cells

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REPORT DATE: May 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual Summary (1 May 2002 - 30 Apr 2003)

4. TITLE AND SUBTITLE
Mechanism of Action of a Novel Analog of Vitamin D3, 1α-hydroxy-24-ethyl Cholecalciferol (D5), in Normal and Transformed Human Breast Epithelial Cells

5. FUNDING NUMBERS
DAMD17-01-1-0272

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plate: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
It is now well established that the active metabolite of vitamin D_3, $1\alpha,25(OH)_2D_3$, regulates cell growth and differentiation in various *in vitro* models. However, its clinical use is precluded due to its hypercalcemic activity *in vivo*. Hence, several less calcemic vitamin D_3 analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models.We have previously reported an analog of vitamin D_3, 1-hydroxy-24-ethyl Cholecalciferol (D5) to be antiproliferative and inducer of differentiation in carcinogen-transformed mouse mammary gland organ culture (MMOC) and breast cancer cells *in vitro* with little or no calcemic activity *in vivo*. We transformed a normal breast epithelial cell line MCF-12F to study the mechanism of action of D5 on the growth of normal *vs* transformed cell lines. Our results showed that D5 was effective in suppressing growth of carcinogen-transformed MCF-12F cells and MMOC, while it does not affect the growth or morphology of normal MMOC or MCF-12F cells. D5 also reduced the expression of EGF receptor in transformed MCF-12F cells and decreased the invasiveness through the Matrigel® coated membranes. Differential gene expression analysis indicated several genes that were altered by D5 treatment in the transformed cells including prohibitin and thioredoxin that were reported to be highly expressed in some tumor tissues. Breast cancer cells that were VDR^+ as well as estrogen receptor positive (ER^+), showed cell cycle arrest and apoptosis, while VDR^+ but ER^- cells showed enhanced expression of various differentiation markers with D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly downregulated in ER^+ BT-474 cells upon D5 treatment. This implies a differential effect of D5 on ER^+ *vs* ER^- cells. Future studies to evaluate the interaction of D5 with ER and other receptors are underway.
(Supported by US Army Research Materiel Command under DAMD17-01-1-0272).

14. SUBJECT TERMS	15. NUMBER OF PAGES
Vitamin D analog; breast epithelial cells; malignant transformation; cell cycle regulation; differential gene expression;	57
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths among women in the US [1]. The active form of vitamin D ($1\alpha,25(OH)_2D_3$ or D_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 D_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 D_3. Our lab has been studying an analog of vitamin D_3, 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 transformed the normal breast epithelial cells MCF-12F 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 *vs* 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, and b) its suitability for use as a prophylactic or therapeutic agent.

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. Consequently, 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 studies to determine possible interaction of D5 with estrogen signalling. 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 included 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 invasiveness 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 second year of award (May 2002 - April 2003) include:

Task 1: Transfection of estrogen receptor alpha (ERα) into MCF-12F cells.
Task 2: Comparison of growth and differentiation of ER^- vs ER^+ cells with or without D5 treatment.
Task 3: Expression of estrogen-inducible genes in MCF-12F cells with or without D5 treatment.

In the previous year we had transformed normal breast epithelial cells, MCF-12F, using two types of carcinogens, dimethylbenz(a)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU). Resulting cell lines were designated MCF-12F$_{DMBA}$ and MCF-12$_{MNU}$, respectively. The transformed cell lines has a growth rate three to four times faster than the parent cell line. We evaluated and compared the growth effects on normal and transformed cells after D5 treatment and determined that D5 inhibits growth significantly in MCF-12F$_{MNU}$ but very modestly in MCF-12F$_{DMBA}$ cells, while it has no affect on MCF-12F cells at 1 μM. We also determined markers of cell proliferation such as PCNA expression and BrdU incorporation in normal *vs* tranformed cells and found these markers to be consistent with the cell growth studies. The growth inhibitory action of D5 on transformed cells does not seem to be brought about by apoptosis as we failed to see apoptotic induction in these cells. Since MCF-12F express very low vitamin D receptor (VDR) and ER, we used SR$^+$ breast cancer cells (BT-474 and MCF-7), to determine possible interaction of D5 with estrogen signaling.

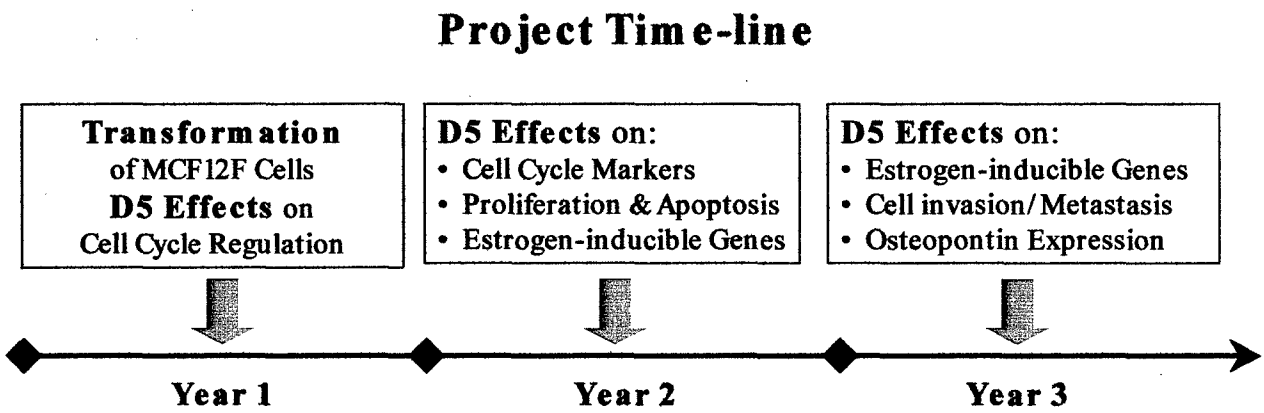

Figure 1: Project time-line for the entire study.

STUDY DESIGN AND METHODS

MCF-12F cells were maintained 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. 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, Westerns, and RT-PCR were used to determine steroid receptor and cell cycle-related protein expression and transcription, respectively, in normal and transformed MCF-12F cells

as well as breast cancer BT-474 and MCF-7 cells. We also used microarrays to determine differential gene expression in MCF-12F and MCF-12F $_{MNU}$ cells. Cell invasiveness was determined by using Matrigel® coated Boyden chambers. Cells were plated on the membranes and percentage of cells migrating through the Matrigel® were counted. Appropriate statistical analysis were performed using Graphpad® Instat software.

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. 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. MCF-12F$_{MNU}$ showed invasion through Matrigel® coated membranes as shown in figure 2. However, upon incubation with D5, the invasiveness of MCF-12F$_{MNU}$ cells decreased.

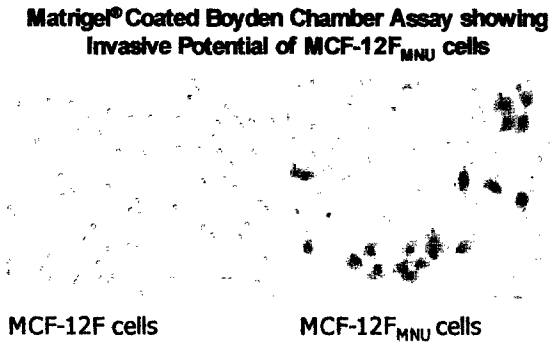

Figure 2. Invasive potential of transformed MCF-12F cells.

Likewise, the transformed cells showed growth inhibition in response to D5 treatment. Specifically, the transformed cells MCF-12F$_{MNU}$ showed cell cycle arrest in G-1 phase of the cycle upon D5 treatment as assessed by flow cytometry. 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-12F$_{DMBA}$	Control	45.1
	D5 (1 μM) *	65.7
MCF-12F$_{MNU}$	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. Although, ER$^+$ breast cancer cells BT-474 showed apoptosis with D5 treatment, the MCF-12F cells, normal and transformed, did not show markers of

apoptosis. To determine the D5 effects on expression of cell cycle regulatory proteins, we performed Western blots on normal and transformed cell lines.

Our results indicated that cyclins A and D1, which play a role in transition of cell cycle from G-1 to S phase were down-regulated in D5 treated MCF-12F$_{MNU}$ cells, but not in MCF-12F or MCF-12F$_{DMBA}$ cells. It should be noted that the growth and invasion inhibitory affect of D5 is more apparent in MCF-12$_{MNU}$ than in the MCF-12F$_{DMBA}$ cells. Additionally, the hypo-phosphorylated form of retinoblastoma gene product was also down-regulated in the MCF-12F$_{MNU}$ cells upon D5 treatment. The expression of another family member of retinoblastoma protein, p107, was likewise reduced in D5 treated MCF-12F$_{MNU}$ cells.

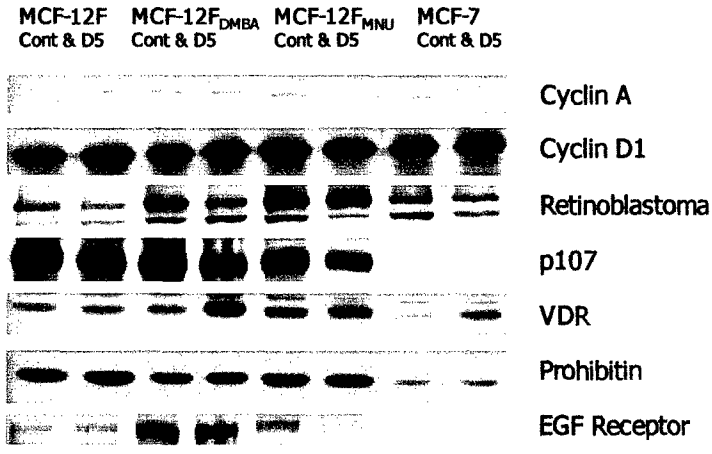

Figure 3. Cell cycle-related protein expression in MCF-12F *vs* transformed cells.

Since the MCF-12F cells express low levels of VDR, we wanted to determine if there is indeed an induction of VDR in MCF-12F$_{MNU}$ cells that respond to D5. Interestingly, there was very modest induction of VDR in MCF-12F$_{MNU}$ cells, while MCF-12F$_{DMBA}$ showed considerable induction of VDR expression. Most likely, VDR induction alone is not sufficient to explain the response to D5 treatment. Some studies have suggested involvement of numerous pathways in effects of vitamin D_3 on breast epithelial cells[6]. MCF-12F cells have low expression of ERα, but show moderate expression of EGF receptor. Upon treatment with D5, no effect on EGF receptor was observed. MCF-12F$_{DMBA}$ showed high levels of EGF receptor that was unaltered by D5 treatment, while MCF-12F$_{MNU}$ expressed moderate levels of EGF receptor that was down-regulated in D5 treated cells (as shown in figure 3). Vitamin D_3 has been shown to down-regulated EGF receptor in colon and other cancer cells, which is followed by growth suppression of these cell lines[7]. Our reports indicate that the D5 decreased expression of EGF receptor in MCF-12F$_{MNU}$ cells that precedes cell cycle arrest of these cells in the G-1 phase.

There fore, 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.

To further evaluate potential D5 target genes, we selected prohibitin and thioredoxin that have been known to express highly in tumor tissues and their role is still very controversial in tumor growth[8,9]. The levels of prohibitin seemed to be somewhat higher in transformed cells than in the parent cell line as determined by immuno-cytochemistry (figure 4) and western blots (figure 5). Prohibitin has been shown to be highly expressed in invasive breast cancer cells, however, there is no direct evidence to support role of prohibitin in invasive potential of breast cancer cells[10].

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

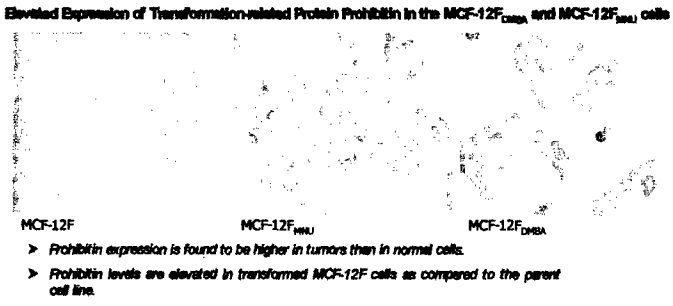

Figure 4. Prohibitin expression in MCF-12F and transformed cells upon treatment with D5.

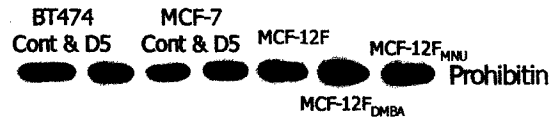

Figure 5. Prohibitin levels in breast epithelial cell lines.

We are currently conducting further studies to determine the role of EGF receptor, prohibitin and thioredoxin in D5 mediated effects in cell growth regulation. As reported earlier, we have tested several breast cancer cell lines for D5 response 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. We also determined the effects of D5 on cell cycle markers on these cell lines.

Our results indicate down-regulation of cell cycle regulatory proteins, such as Cyclins A and D1, and cyclin dependent kinase 2 (CDK-2) as determined by Western blots (shown in figure 6). The CDK inhibitors p21^{wafl} and p27^{kipl}, on the other hand, were up-regulated by D5 treament in BT-474 and MCF-7 breast cancer cells, indicating mechanism of growth arrest induced by D5. Furthermore, an S-phase related transcription factor E2F-1 was also modestly down-regulated by D5 treatment as shown in figure 7. We had previously reported decrease in estrogen-inducible progesterone receptor upon D5 treatment in BT-474

cells. The ER itself did not seem to be effected by D5 treatment in BT-474 cells. Therefore, we used high ER expressing MCF-7 cells to look at effect of D5 on ER. Western blots showed modest down-regulation of ER upon prolonged treatment with D5 (shown in figure 8).

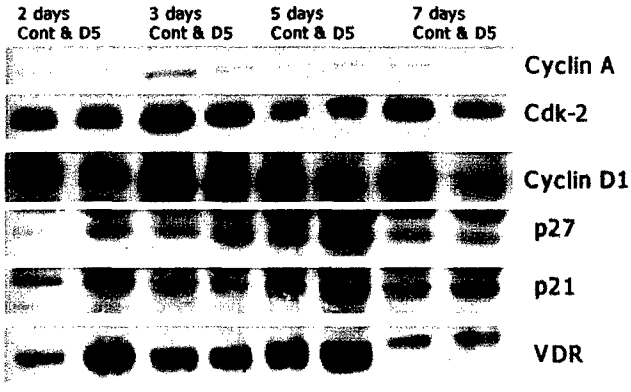

Figure 6. Expression of cell cycle markers in BT-474 cells.

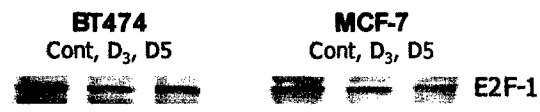

Figure 7. Expression of S-phase transcription factor E2F-1.

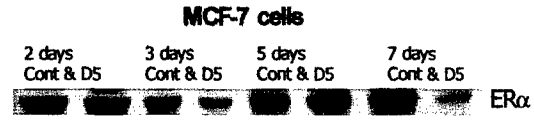

Figure 8. Expression of ER in MCF-7 cells.

To investigate further whether D5 would effect expression of ER in BT-474 cells, we performed RT-PCR in D5 treated BT-474 cells with or without added estradiol. The ERα transcription decreased in response to D5, while ERβ was unaffected (figure 9). It is possible that D5 modestly affects the ER α levels that were not detectable in Western blot analysis. Results from lab indicate that D5 might be working through suppression of ERα as well as EGF receptor-mediated growth in these cell lines. We wanted to identify additional targets of D5 in SR+ breast cancer cells. Therefore, we used Human UniGene 10K arrays. The differential gene expression profiles were determined for BT-474 cells with or without D5 treatment. Consistent with our previous results, 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.

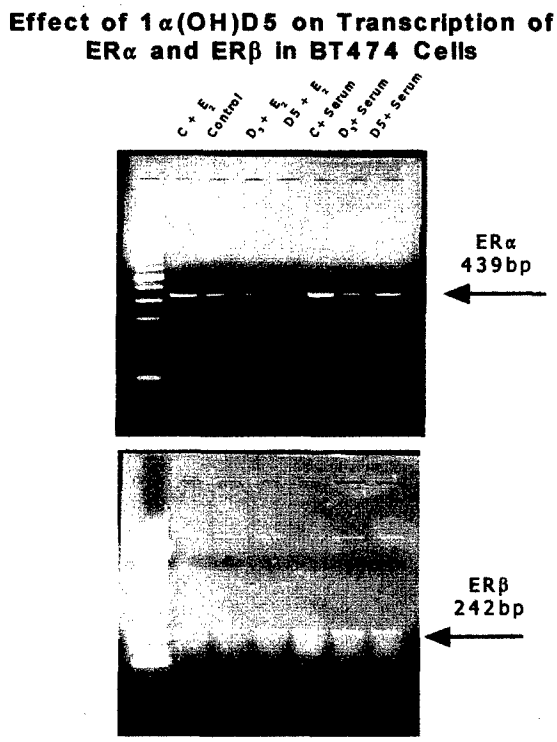

Figure 9. Transcription of ERα upon treatment with D5.

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 ↑	$p < 0.01$

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 ER as well as 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 [11]. Moreover, new targets of D5 are implicated in our results that might render it useful in SR^- and invasive breast cancer cells as well. Currently, we are in the process of generating ER and VDR transfected cell lines, which may clarify the interaction of D5 with steroid signalling pathways. In the final 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).

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 ERα and estrogen-inducible genes with D5 treatment in SR$^+$ breast cancer cells (BT-474 and MCF-7).
- D5 effects on cell cycle regulatory proteins using MCF-12F cells as well as BT-474 and MCF-7 cells.
- Identification of other potential target genes of D5 in breast epithelial cells, such as prohibitin, thioredoxin, and EGF receptor.

REPORTABLE OUTCOME

Manuscript: Efficacy and Mechanism of Action of 1α-hydroxy-24-ethyl Cholecalciferol in Breast Cancer Prevention and Therapy. **Erum A. Hussain**, Rajeshwari R. Mehta, Tapas K. Das Gupta, Rajendra G. Mehta. (Accepted for publication in *Recent Results in Cancer Research*).

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*, 2003; 523-524: 253-64.

Abstracts: Efficacy and mechanism of action of 1α-hydroxy vitamin D5 in breast cancer. Rajendra G. Mehta, Rajeshwari R. Mehta, **Erum A. Hussain**, D.L. McCormick, Robert M. Moriarty, and Tapas K. Das Gupta. Vitamin D Analogs in Cancer Prevention and Therapy, !st Symposium, Homburg/SAAR, Germany. May, 2002.

Vitamin D analog, 1 α-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, San Francisco, CA. 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.

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APPENDICES

Manuscript 1: Efficacy and Mechanism of Action of 1 α-hydroxy-24-ethyl Cholecalciferol in Breast Cancer Prevention and Therapy. **Erum A. Hussain**, Rajeshwari R. Mehta, Tapas K. Das Gupta, Rajendra G. Mehta. (Accepted for publication in *Recent Results in Cancer Research*).

Manuscript 2: Chemoprevention of Mammary Carcinogenesis by 1 α(OH)D5, a Synthetic Analog of Vitamin D. Rajendra G. Mehta, **Erum A. Hussain**, Rajeshwari R. Mehta, and Tapas K. Das Gupta. *Mutation Research*, 2003; 523-524: 253-64.

Curriculum Vitae

Efficacy and Mechanism of Action of 1α-hydroxy-24-ethyl-Cholecalciferol (1α[OH]D5) in Breast Cancer Prevention and Therapy

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Keywords:- vitamin D analog, breast cancer, chemoprevention

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Abstract

It is now well established that the active metabolite of vitamin D_3, 1α,25$(OH)_2D_3$, regulates cell growth and differentiation in various *in vitro* cancer models. However, its clinical use is precluded due to its hypercalcemic activity *in vivo*. Hence, several less calcemic vitamin D analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models. A novel analog of vitamin D_3, 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5), has currently been under investigation in our laboratory for its application in breast cancer prevention and therapy. 1α(OH)D5 had been shown to inhibit development of estrogen and progesterone dependent ductal lesions as well as steroid hormone independent alveolar lesions in mammary gland organ culture (MMOC) model. Moreover, the inhibitory effect was more significant if 1α(OH)D5 was present during the promotional phase of the lesion development.

The growth inihibitory effect of 1α(OH)D5 has also been manifested in several breast cancer cell lines, including BT-474 and MCF-7. Breast cancer cell lines that responded to 1α(OH)D5 treatment were vitamin D receptor positive (VDR+). Vitamin D receptor negative (VDR–) cell lines, such as MDA-MB-231 and MDA-MB-435, did not show growth inhibition upon incubation with 1α(OH)D5. This suggests requirement of VDR in 1α(OH)D5 mediated growth effects. Interestingly, breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+), showed cell cycle arrest and apoptosis, while VDR+ but ER– cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α(OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly downregulated in ER+ BT-474 cells with 1α(OH)D5 treatment. This implies a differential effect of 1α(OH)D5 on ER+ *vs* ER– cells. Additionally, comparison between the effects of 1α(OH)D5 on normal *versus* transformed cells indicated that 1α(OH)D5 does not suppress cell proliferation of normal epithelial cells but selectively targets growth of transformed cells.

We extended our experiments to determine *in vivo* effects of 1α(OH)D5 using MNU-induced mammary carcinogenesis model in female Sprague-Dawley rats. Results showed that 1α(OH)D5 (25-50 μg/kg diet) decreased the incidence and multiplicity of mammary tumors in these rats. In addition, it increased the latency period of early precancerous lesions. Similar studies, with DMBA as a carcinogen in younger rats, showed that 1α(OH)D5 supplementation

was effective in reducing onset of carcinogenesis in rats and the effect was largely reflected during the promotional phase of carcinogenesis. Recently, a preclinical toxicity profile for 1α(OH)D5 was completed in rats and dogs that provides estimation of the maximum tolerated dose in mammals. Based on our findings, we will shortly be initiating a 1α(OH)D5 phase I clinical trial for breast cancer patients.

Introduction

Breast cancer is generally characterized by transformation of normal to an atypical hyperplastic epithelium with subsequent risk of progression to intra-ductal carcinoma and in some cases invasion into stroma (Mallon *et al*, 2000). Breast cancer is the second leading cause of cancer related deaths among women in the US, with about 180,000 new cases and 46,000 deaths annually (Edwards *et al*, 2002). Although the overall incidence of breast cancer has not been reduced in the last decade, the breast cancer related mortality has been decreasing with approximately 3.4% annual decrease from 1995 through 1998 in the US (Howe *et al*, 2001; Peto *et al*, 2000). This decrease in mortality is probably a result of availability of greater screening efficiency and better chemopreventive and therapeutic strategies. Despite increased survival rates, breast cancer results in considerable morbidity and patient care costs. Chemoprevention is an important aspect of curbing the progression or recurrence of the disease. The chemopreventive agents usually include natural or synthetic compounds that can either prevent transformation or inhibit proliferation of transformed cells by inducing apoptosis, growth arrest or differentiation of initiated and transformed cells (Rosenbaum-Smith & Osborne, 2000). Several classes of compounds have been under investigation in this regard. These include, selective estrogen receptor modulators, retinoids, deltanoids (vitamin D derivatives), phytoestrogens, flavonoids, and aromatase inhibitors among others (Kelloff *et al*, 1996).

On a global basis, breast cancer incidence is five fold higher among middle-aged women in the Western countries than the women from Asian countries. Various diet and lifestyle as well as genetic factors have been implicated in the high occurrence of breast cancer in the Western world. Some epidemiological studies have shown association of lower sunlight exposure to higher breast, colon, and prostate cancer mortality rates in the

US and other Western countries (Freedman *et al*, 2002; Polek & Weigel, 2002; Garland *et al*, 1990; Gorham *et al*, 1990). This is consistent with reports of an association of breast cancer mortatlity with lower serum vitamin D_3 levels (John *et al*, 1999; Christakos, 1994). Lower serum vitamin D_3 levels could be due to lower sunlight exposure as well as lower dietary intake.

The biologically active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ or calcitriol, is a steroid hormone that was identified in the early 1920s as an anti-rachitic substance (Carpenter & Zhao, 1999). Later it was established that vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol by the action of ultra-violet radiation. Vitamin D_3 is activated subsequently in liver and kidney by the hydroxylation reactions at C25 and 1α positions to yield $1\alpha,25(OH)_2D_3$. Calcitriol has been known to exert calciotrophic effects mainly through increasing calcium uptake in the intestine for regulation of bone health. Aside from its role in calcium homeostasis, vitamin D_3 is involved in regulation of various cellular processes. Vitamin D_3 binds to nuclear vitamin D receptor (VDR) that undergoes conformational changes, which allow VDR to function as a transcription factor (Jones *et al*, 1998; Haussler, 1986). Earlier, the VDR was found to be present in abundance in intestine, bone, liver, and kidney cells. Aside from the classical target organs, the VDR has now been isolated from a variety of tissues including normal mammary epithelium as well as breast tumors (Buras *et al*, 1994; Eisman *et al*, 1980).

In order for VDR to function, it needs to interact with vitamin D response elements (VDRE) and bind to DNA to initiate or repress transcription (Pike, 1991). VDR must form a dimer to stabilize VDRE transactivation (Jones *et al*, 1998). Most common partners for VDR heterodimerization are nuclear accessory factor (NAF) and retinoid X receptor (RXR) (Rachez & Freedman, 2000). VDR transactivation of VDRE results in regulation of a wide variety of genes, some of which are involved in cell growth and proliferation. Vitamin D_3 also exerts some non-genomic rapid responses possibly through a putative membrane receptor (Falkenstein *et al*, 2000).

The presence of VDR in the normal mammary epithelial cells suggests role of calcitriol in the regulation of mammary gland function. The levels of VDR in mammary tissue increase during pregnancy and lactation and decrease as the glands regress back to normal size (Zinser *et al*, 2002; Narvaez *et al*, 2001). VDR knockout mice have been

shown to have larger mammary gland than normal mice and that the glands would not regress back to prepregnancy size at the termination of lactation (Zinser *et al*, 2002). This suggests that vitamin D mediated signalling may be very important for maintaining the normal cycling of the mammary gland. Various case studies indicate that a high percentage (60-80 %) of breast cancer epithelia contain VDR and that there is a positive correlation between VDR polymorphisms and increased risk of breast cancer (Bretherton-Watt *et al*, 2001; Lundin *et al*, 1999). These reports further signify vitamin D_3 mediated signalling to be of importance in regulation of healthy mammary gland. In cell culture models, vitamin D_3 has been demonstrated as an inducer of growth arrest and differentiation in various cancer cell lines, including breast cancer cells (Welsh *et al*, 1998; James *et al*, 1997). Taken together, these results warrant potential use of vitmain D_3 in cancer prevention and therapy. However, due to its hypercalcemic activity, vitamin D_3 can not be administered at doses that would be effective for chemoprevention or therapy. Adverse effects of vitamin D_3 at cancer preventive doses are hypercalcemia, soft tissue calcification, weight loss, and possibly death (Roder & Stair, 1999; Vieth, 1999).

Since the early 1980s, there has been a search for vitamin D_3 analog that would selectively modulate VDR to produce growth regulating effects without interfering with the calcium metabolism. Several analogs have been synthesized and tested for this purpose; but only a few have shown promising results in cell culture and animal models. Vitmain D_3 analogs currently being evaluated for breast cancer prevention include, Seocalcitol (EB-1089), Calcipotriol (KH-1060), Maxacalcitol (OCT), RO-24-5531, and 1α(OH)D5 (Mehta & Mehta, 2002; Guyton *et al*, 2001). In this review, we are summarizing the results from experiments conducted in our laboratory that elucidate the potential role of 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5) in breast cancer prevention or therapy.

Synthesis and Characterization of Vitamin D Analog, 1α(OH)D5

As mentioned earlier, vitamin D_3 can be obtained from food as well as synthesized in the skin through the action of sunlight. Vitamin D_3 belongs to the family

of 9,10-secosteroids which differ only in side chain structure (Napoli *et al*, 1979). Other forms of D-compounds include, D2, D4, D5, and D6. In the late 1970s, major interest in the synthesis of these compounds was to evaluate them for use in management of renal osteodystrophy and osteoporosis. In this regard the calcemic activity of D series of compounds was compared and D5 was found to be the least calcemic of all (Napoli *et al*, 1979); a property that would later serve useful in its possible application for cancer prevention. The D5 form is also known as irridated 7-dehydrositosterol. The hydroxylated form of D5 (1α[OH]D5) was synthesized as described previously (Mehta *et al*, 1997a).

Figure I

Briefly, β-sitosterol acetate was converted to 7-dehydro-β-sitosterol acetate by allelic bromination and dehydrobromination. Lithium aluminum hydride and tetrahydrofurane were used to reduce 7-dehydro-β-sitosterol to 7-dehydro-3β-sitosterol. The reaction mix was sequentially subjected to photolysis and thermolysis to yield 24-ethyl-cholecalciferol (D5). D5 was hydroxylated by Paaren-DeLuca hydroxylation sequence to produce 1α(OH)D5. The product was crystallized and characterized by ^{1}H nuclear magnetic resonance at 400 Hz and mass spectroscopy. The purity was assessed by high pressure liquid chromatography. The following properties were observed: melting point = 150-152°C; UV λ-max = 265 nm; molar extinction coefficient (ε) = 18000; molecular weight = 428.7. The major structural differences between biologically active vitamin D_3 and 1α(OH)D5 are the lack of hydroxylation at the C-25 position and the presence of an ethly group at the C-24 position in the 1α(OH)D5 molecule (figure I).

Calcemic Activity of 1α(OH)D5

Earlier studies in Dr DeLuca's lab had shown that among the known vitamin D series of compounds (vitamin D2 to D6), D5 is the least calcemic of all (Napoli *et al*, 1979). D5 was found to be 80-fold less active than vitamin D_3 in the intestine and about 100 to 200-fold less active in bone in mobilizing the Ca^{++} stores (Napoli *et al*, 1979). The calcemic activity of the hydroxylated form was not known. Therefore, we measured

calcemic activity as well as body weight change in animal models to determine the maximum tolerable dose and toxicity of 1α(OH)D5. In the first experiment, three week old Sprague-Dawley male rats were fed vitamin D_3 free diet containing 0.47 g calcium and 0.3 g phosphorus/100 g diet (Mehta *et al*, 1997a). These rats were kept under yellow light to create vitamin D_3 deficiency state. After the rats were fed vitamin D_3 deficient diet for three weeks, their plasma calcium levels were measured and rats with calcium levels < 6.0 mg/dL were considered vitamin D_3 deficient. Vitamin D_3 deficient rats were administered 1α(OH)D5 intragastrically for 14 days and the plasma calcium levels were measured. The control group showed a plasma calcium concentration of 5.4 + 0.3 mg/dL, while the rats receiving 1α(OH)D5 at a dose of 0.042 μg/kg/day had plasma calcium concentration of 6.0 + 0.63 mg/dL which was not significantly different from the control (Mehta *et al*, 1997a). On the other hand, vitamin D_3 increased plasma calcium concentration 50 % over that of the control group (table 1). During these experiments, the 1α(OH)D5 group did not differ in total body weight from control group. No other signs of toxicity were observed in 1α(OH)D5 fed rats compared to control.

In a separate experiment, female Sprague Dawley rats were fed diet supplemented with 1α(OH)D5 to determine its calcemic activity in vitamin D_3 sufficient rats. Food was provided *ad libitum*. There was no body weight change at 50 μg 1α(OH)D5/kg diet in vitamin D_3 sufficient rats while a dose of 12.8 μg 1α,25$(OH)_2D_3$/kg diet was sufficient to bring about significant weight loss in the animals (table 1). Maximum tolerated dose was determined to be 50 μg/kg diet, based on the weight and calcemic activity of 1α(OH)D5 in these rats (Mehta *et al*, 2000a). In addition to these experiments, we also conducted toxicity studies under the GLP using rats and dogs. For rats, the dose at which signs of toxicity first appeared was 100 μg 1α(OH)D5/kg diet which is twice the amount needed to bring about effective chemoprevention. However, the dogs had much lower tolerance for 1α(OH)D5 as compared to rats. Based on these results we are now conducting further studies to determine appropriate and safe dose of 1α(OH)D5 for use in clinical settings.

Table 1

Since vitamin D_3 exerts most of its affects through binding to VDR, we evaluated the ability of 1α(OH)D5 to bind to VDR. The 1α(OH)D5 to VDR association experiments were conducted in Dr Rahul Ray's lab (unpublished data). Results showed that the binding affinity of 1α(OH)D5 in competition with $1\alpha(OH)_2D_3$ to VDR is 1000-fold less (figure II). The IC_{50} for 1α(OH)D5 was 100 pM while for $1\alpha(OH)_2D_3$, it was 0.08 pM. The lower binding affinity may explain the decreased calcemic activity of 1α(OH)D5. However, due to its lower calcemic activity, 1α(OH)D5 can be administered at much higher doses than $1\alpha(OH)_2D_3$. Moreover, lower 1α(OH)D5 affinity to VDR in comparison to $1\alpha,25(OH)_2D_3$ would assure non-interference of 1α(OH)D5 with the normal vitamin D_3 metabolism even at high doses. This quality can allow use of 1α(OH)D5 for prevention in the gneneral population as well as high risk groups. It is also important to note that the *in vivo* VDR affinity to its ligand is tissue specific (Napoli *et al*, 1979), which could not be manifested in our experiments that were conducted using purified VDR.

Figure II

Anticarcinogenic Effects of 1α(OH)D5 in *in vitro* Models

The effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland (MMOC). The mammary glands form balb/c mice are harvested and cultured in presences of appropriate hormones (Mehta *et al*, 1997b). These glands are subjected to short stimulation with a carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA), which results in formation of precancerous preneoplastic lesions. When implanted in syngeneic hosts, the epithelial cells from these lesions give rise to adenocarcinomas. Effective chemopreventive agents would inhibit the development of these preneoplastic lesions. The chemopreventive activity of a compound in MMOC correlates very well with the activity in *in vivo* carcinogenesis models (Mehta *et al*, 1997b). Using DMBA-induced MMOC model, Mehta *et al* (1997a) showed that 1α(OH)D5 possesses chemopreventive activity. Fifteen mammary glands (per group) from balb/c mice were incubated with appropriate hormones and were exposed to the

carcinogen DMBA (2 μg/mL of culture media) on day three of a 24-day culture. The group of glands incubated with 1α(OH)D5 showed significant reduction of lesion formation as compared to the control group (figure III). Percent inhibition of lesion formation in each treatment group was calculated by comparing the incidences of lesions between the control and the treated group. A dose response curve showed that 100 % inhibition was achieved at 10 μM 1α(OH)D5 concentration, but the optimal dose seems to be 1 μM as it shows significant (75 %) inhibition without any signs of cytotoxicity. Vitamin D_3, on the other hand, caused dilation of ducts and disintegration of alveolar structures as signs of toxicity at 1 μM concetration. Based on the MMOC model, 1 μM 1α(OH)D5 seems to be equivalent in potency to 0.1 μM 1α,25$(OH)_2D_3$.

Figure III

In order to establish the stage specificity for the effectiveness of 1α(OH)D5 in DMBA-induced MMOC model, 1α(OH)D5 was added either prior to or subsequent to carcinogen treatment (Mehta *et al*, 2002a). All the glands were incubated with growth promoting hormones, insulin, prolactin, aldosterone, and hydrocortisone, that resulted in formation of alveolar structures. The glands were incubated with DMBA on third day of 24-day culture. The glands were divided into four groups with 15 glands per group. The control group received only the vehicle, while initiation and promotion group received 1α(OH)D5 for the first 10 days of culture. Inititation only group received 1α(OH)D5 for first four days of culture, whereas promotion only group received the treatment after withdrawal of carcinogen (days 4-10). After the initial 10 days, the culture was incubated with insulin alone to allow the regression of mammary structures. At the end of day 24, the glands were terminated for analysis. Results indicated that 1α(OH)D5 is more effective when present during the promotional stages of lesion formation (Mehta *et al*, 2002a).

In addition to inhibition of lesion formation, 1α(OH)D5 was effective in inducing VDR and TGFβ1 expression in mammary epithelial cells of MMOC. VDR and TGF-β1 expression was measured using immuno-histochemistry. Briefly, paraffin embedded sections were rehydrated, fixed, permiabilized, and incubated with primary antibody. The

primary antibody binding was detected using biotinylated link and peroxidase conjugated streptavidin, which was then visualized by 3-amino-9-ethyl-carbazole as chromogen. The mammary epithelial cells which stained negative for VDR failed to show TGF-β1 induction upon 1α(OH)D5 treatment. This implies the involvement of VDR in 1α(OH)D5 mediated effects. The extent of induction of VDR and TGF-β1 upon treatment with 1.0 μM 1α(OH)D5 was similar to that observed with 0.1 μM vitamin D_3 (Mehta *et al*, 1997a). Despite the 1000-fold lower affinity of 1α(OH)D5 for VDR in comparison to 1α,25$(OH)_2D_3$, its chemopreventive activity is equivalent to 1α,25$(OH)_2D_3$ at only a 100-fold higher concentration. Therefore, it seems likely that the antiproliferative effects of 1α(OH)D5 may not be dependent solely upon its *in vitro* interactions with VDR.

Since the MMOC experiments involved the whole organ, the actions of 1α(OH)D5 on breast epithelia itself were not clearly established. Hence, we tested the growth effects of 1α(OH)D5 on various breast cancer cell lines of epithelial origin. All the cell lines tested were purchased from ATCC except UISO-BCA-4 cells. This cell line was established in our laboratory from metastatic pleural fluid obtained from a 56-year old woman with a confirmed diagnosis of breast carcinoma (Mehta *et al*, 1992). The growth effects of 1α(OH)D5 were assessed on BT-474, MCF-7, ZR-75-1, T-47D, MDA-MB-231 and MDA-MB-435 cell lines using multiple measures; cell counter, MTT absorbance assay (Twentyman & Luscombe, 1987), and cell cycle analysis with propidium iodide staining and flow cytometry (Vindelov *et al*, 1983). The overall effects of 1α(OH)D5 on growth of different cell lines are summarized in table 2. All the cell lines that were positive for VDR showed significant growth inhibition ($p < 0.05$) after 72 hours of incubation with 1α(OH)D5. BT-474 and MCF-7 (VDR+ ER+ PR+) cells showed the greatest growth inhibition and G-1 cell cycle arrest upon 1α(OH)D5 treatment. Similarly, UISO-BCA-4 (VDR+ ER– PR–) cells exhibited growth inhibition in response to 1α(OH)D5 treatment. On the other hand, VDR– MDA-MB-231 and MDA-MB-435 cells did not show any growth inihibition at 1 μM 1α(OH)D5 treatment (Mehta *et al*, 2002b). The dose response curve for 1α(OH)D5 effect in BT-474 cells was similar to that observed in the MMOC experiments.

Table 2

Chemopreventive Efficacy of 1α(OH)D5 in *in vivo* Carcinogenesis Models

Once we established the *in vitro* efficacy of 1α(OH)D5, the effects of 1α(OH)D5 were evaluated in experimental mammary carcinogenesis models. We used mammary specific carcinogen N-methyl-N-nitrosourea (MNU) in rats to induce tumors and evaluated the efficacy of 1α(OH)D5 to prevent or delay the incidence of mammary cancers in these rats (Mehta *et al*, 2002a). Fifteen Sprague-Dawley female virgin rats per group (9 weeks old) were fed 1α(OH)D5 supplemented diet (25 or 50 μg/kg) for two weeks before the carcinogen treatment. The carcinogen MNU was given as a single intravenous injection of 50 mg acidified MNU/kg body weight at 80 days of age. The rats continued to receive the 1α(OH)D5 supplemented diet until they were sacrificed at 190 days of age. The tumor incidence in control rats was 80 %, which compared to control, decreased in 25 and 50 μg/kg diet group by 33 % and 42 %, respectively (table 3). The tumor incidence in the low dose group was not significantly reduced from control (p = 0.12), whereas the high dose group had significantly lower tumor incidence (p = 0.03). However, when the three groups were compared together using log-rank analysis, the comparison reached statistical significance (p = 0.0495). The tumor multiplicity was not significantly different between the control and the 25 μg/kg diet group, but it was significantly lower in the high dose group (p = 0.02).

The encouraging results from MNU-carcinogenesis model prompted us to extend our *in vivo* experiments. Since MNU is a direct acting carcinogen, we chose another mammary specific carcinogen that needs to be metabolized, such as DMBA. For the DMBA carcinogenesis study, seven weeks old rats (20 per group) were given 15 mg DMBA intragastrically. 1α(OH)D5 was supplied in the diet (20-40 μg/kg diet) two weeks prior to carcinogen treatment. Control group showed 85 % tumor incidence and high dose group showed 60 % incidence, while the low dose group showed significant

decrease in incidence (40 %). Table 3 summarizes the results from *in vivo* experiments. Although the high dose group did not show significant decrease in tumor incidence, it had significantly lower tumor multiplicity (0.6 compared to 1.9 in control group). Moreover, the chemopreventive efficacy of 1α(OH)D5 was more pronounced when provided at progressional stages of the disease.

Table 3

In addition, xenograft of UISO-BCA-4 cells pretreated with 1 μM 1α(OH)D5 for 10 days failed to form tumors in athymic (4 weeks old) mice (Mehta *et al*, 2002b). In other studies, UISO-BCA-4 cells were orthotopically xenografted in athymic mice and either 8 ng 1α(OH)D5 per animal was injected IP thrice a week or 1α(OH)D5 was provided in the diet at 12.5 μg/kg diet for six weeks. All the animals in the control group formed tumors whereas only 1 of the treated animals showed a scab like structure at injection site in the IP group. Forty percent of control showed metastasis to lymph nodes but 1α(OH)D5 treatment prevented metastasis of cells transplanted in athymic mice (Mehta *et al*, 2002b). In the dietary treatment group, 1α(OH)D5 inhibited growth of UISO-BCA-4 cells and the tumor volume was suppressed to nearly 50 % of control. Similar results were obtained with BT-474 xenograft in athymic mice. These results suggest that 1α(OH)D5 induced cell growth inhibition and differentiation is protective against tumor growth in the xenograft model as well.

Growth Response of Normal *vs* Transformed Cells to 1α(OH)D5

While we established that 1α(OH)D5 has growth inhibitory action on cancer cells, the effects on normal breast epithelial cells were not known. In order to determine that, we cultured mammary glands from mouse with appropriate hormones in the absense of any carcinogens. Ten glands were treated with 1α(OH)D5 and other glands were used as controls. At the end of six day culture, the glands were terminated, paraffin embedded, and sectioned for pathological evaluation. Histopathological examination showed no

difference in the growth and morphology of glands treated with 1α(OH)D5 from that of control. In view of that, we evaluated effects of 1α(OH)D5 on MCF-12F cells, which are non-tumorigenic breast epithelial cells derived from reduction mammoplasty from a 60 year old caucasian woman. These cells were spontaneously immortalized by longterm culture in low Ca^{++} media. To determine their growth response, MCF-12F cells were incubated with 1α(OH)D5 for various intervals, but no growth inhibitory effect was observed at 1μM concentration.

To establish selectivity of 1α(OH)D5 effects on transformed or preneoplastic cells, we transformed MCF-12F cells with DMBA and MNU to study if the transformation status could affect the response to 1α(OH)D5. Transformation was performed using the protocol described elsewhere (Lazzaro *et al*, 1997). Briefly, passage 10 MCF-12F cells were grown to subconfluency in tissue culture dishes and incubated with DMBA (2 μg DMBA/mL culture media) for 24 hours. The procedure was repeated the next day. Extensive cell death resulted. The surviving cells were allowed to grow in fresh medium and later selected out with serum starvation. The resulting cell line was designated MCF-$12F_{DMBA}$. Similarly, in another experiment, MNU was dissolved in acidified saline (pH 5.3) and added to subconfluent MCF-12F cells at a concentration of 25 μg/mL twice daily for two days. The surviving cells were allowed to grow and new cell line was established after serum starvation. These cells were called MCF-$12F_{MNU}$. Growth rate and morphological characteristics were compared between these cell lines. The growth rates of transformed cells were thrice as much as MCF-12F. By the fifth passage post carcinogen treatment, the MCF-$12F_{DMBA}$ doubling time was reduced to one third of MCF-12F while for MCF-$12F_{MNU}$, it was reduced to one fourth of MCF-12F. Moreover, the transformed cell lines did not exhibit contact inhibition which is characteristic of the normal cells.

As mentioned earlier, MCF-12F cells showed no growth inhibitory response with 1α(OH)D5 treatment. The transformed cells, on the other hand, showed significant growth inhibition (60 % for MCF-$12F_{MNU}$ and 40 % for MCF-$12F_{MNU}$) as determined by the MTT absorbance assay. Other measures of growth provided similar results (table 4).

These studies indicate that the transformed cells respond differently to 1α(OH)D5 treatment than the parent cell line.

Table 4

Potential Mechanism of Action of 1α(OH)D5 in Breast Cancer Prevention and Therapy

Previously mentioned studies have implicated the involvement of VDR in 1α(OH)D5 mediated growth effects. VDR– highly metastatic cells, such as MDA-MB-231 and MDA-MB-435, do not respond to 1α(OH)D5 treatment. Moreover, mammary epithelial cells which lack VDRs also fail to respond to 1α(OH)D5 and do not show induction of VDR and TGF-β1 (Mehta *et al*, 1997a). VDR+ breast cancer cells, such as T-47D, had been shown to increase transcription of VDR upon incubation with 1α(OH)D5 as determined by RT-PCR (Lazzaro *et al*, 2000). This VDR induction was not observed in the cell line BT-474 either at transcription or expression levels upon treatment with 1α(OH)D5. A possible explanation could be the high constitutive levels of VDR present in this cell line. To ascertain VDR mediated VDRE transactivation activity of 1α(OH)D5, we used the CAT reporter gene containing VDRE (VDRE-tk-CAT). For this purpose, CV-1 monkey renal cancer cells were used as these lack a functional VDR. After VDRE-tk-CAT transient transfection into CV-1 cells, 1α(OH)D5 could not induce the CAT activity in these cells. But when the cells were cotransfected with VDRE and VDR, there was an enhanced expression of CAT activity suggesting capability of 1α(OH)D5-to activate VDR mediated signalling. The relative CAT activity in CV-1 cells that had been cotransfected with VDRE and VDR was 200,000 fold higher than control when treated with 0.1 μM 1α(OH)D5 (Lazzaro *et al*, 2000).

Breast cancer UISO-BCA-4 cells are ER– and PR–, but VDR+. These cells responded differently to 1α(OH)D5 than the ER+ cells (Mehta *et al*, 2002b). UISO-BCA-4 cells were treated with 0.1μM 1α(OH)D5 for 10 days. The 1α(OH)D5 treatment

resulted in induction of intracytoplasmic casein granules, increased lipid droplets, ICAM-1, α2-integrin, nm23, and VDR; manifesting the differentiation markers. Use of this cell line allows us to determine estrogen-independent effects of 1α(OH)D5. While 1α(OH)D5 induced differentiation in ER– cells, it induced apoptosis in ER+ BT-474 and MCF-7 cells as determined by acridine orange/ethidium bromide staining and TUNEL assay (Mehta *et al*, 2002b). In both these cell lines there is a G-1 cell cycle arrest followed by apoptosis.

Because the actions of 1α(OH)D5 differ in ER+ breast cancer cells, we examined the effects of 1α(OH)D5 on estrogen dependent signalling in the the ER+ PR+ BT-474 cells. BT-474 cells showed down-regulation of both ER and estrogen inducible PR transcription upon treatment with 1α(OH)D5 as determined by RT-PCR (figure IV). This was in turn followed by down-regulation at the expression level as estimated by immuno-cytochemistry (figure V). These results are consistent with reports by other researchers that describe role of vitamin D_3 in down-regulation of estrogen inducible genes (Swami *et al*, 2000; Stoica *et al*, 1999). Perhaps vitamin D_3-VDR pathway may be a negative feedback mechanism to regulate the estrogen induced proliferation of the mammary tissue. Some researchers have postulated interaction of VDR-D_3 to ERE to repress the estrogen mediated gene transcription (Welsh *et al*, 1998; Demirpence *et al*, 1994).

Figure IV

Figure V

Since vitamin D_3 is known to regulate a wide variety of genes, we investigated other potential gene targets of 1α(OH)D5 in BT-474 cells. The microarray was performed using Human UniGene 1 by Incyte™ Genomics, Inc., that contained 8,000 genes along with appropriate controls. Among the major targets of 1α(OH)D5 were the estrogen inducible genes PR, trefoil factor 1 (pS2), and trefoil factor 3 ($p < 0.05$). A few selected genes that were statistically significantly altered are presented in table 5.

As mentioned earlier, the transformed MCF-12F cells showed growth inhibition even though these cells express very low levels of steroid receptors. It is possible that

other mechanism are at work to bring about growth arrest in MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$ cells. Therefore, we used Clontech Atlas™ microarrays with 10,000 genes to identify differentially expressed genes in the transformed MCF-12F$_{MNU}$ cells as compared to the MCF-12F parent cell lines. In a second comparison, we assessed the genes differentially expressed by 1α(OH)D5 treatment in MCF-12F$_{MNU}$ cells. Interestingly, many genes that were differentially expressed in MCF-12F$_{MNU}$ cells compared to the MCF-12F cells were altered inversely in 1α(OH)D5 treated MCF-12F$_{MNU}$ cells (table 5). Most of the genes that were effected were transcriptiopn related and mitochondrial genes. Of interest are proteins, such as vimentin, prohibitin, MAPK-7, and HSP-27, which are usually expressed at higher levels in mammary tumors (Atanaskova *et al*, 2002; Zajchowski *et al*, 2001; Storm *et al*, 1996). These proteins were down-regulated in 1α(OH)D5 treated cells. Differentiation related proteins, such as integrins and cadherins were upregulated by 1α(OH)D5 in both BT-474 and MCF-12F$_{MNU}$ cell systems.

Table 5

Prohibitin might be a potentially important vitamin D_3 regulated protein which was found to be highly expressed in the transformed MCF-12F cells than the parent cell line (data not shown). Some studies have shown high prohibitin levels in tumor tissue and cancer cell lines (Jupe *et al*, 1996; Asamoto & Cohen, 1994). However, the role of this mitochondrial protein is controversial. Wang and coworkers (1999) have shown its involvement in regulation of cell cycle, whereas, others have shown that the levels do not repersent the cell cycle related functions but rather are indicative of mitochondrial stress (Coates *et al*, 2001). It is possible that the mitochondrial stress may be indicative of the higher proliferative rates of the transformed cells. Another protein of interest was thioredoxin, which was upregulated in MCF-12F$_{MNU}$ cells and downregulated by 1α(OH)D5 treatment. Thioredoxin is a redox protein with growth factor activity that modulates the activity of several proteins important for cell growth. Some researchers have observed increased thioredoxin transcription and expression in priumary human tumors (Matsutani et al, 2001; Berggren et al, 1996). Administration of inhibitors of

thioredoxin system has been shown to have anti tumor acitvity *in vivo* (Kirkpatrick et al, 1999). Moreover, Gallegos and coworkers (1996) reported that transfection of dominant-negative mutant thioredoxin resulted in reversal of transformed phenotype of human breast cancer cells. Therefore, it appears that the mechanism of action of 1α(OH)D5 involves multiple genes and pathways, some of which have not yet been thoroughly investigated. Further studies are needed to elucidate the mechanism of action of 1α(OH)D5 in normal and cancer breast cells.

Conclusions

In conclusion, our studies on 1α(OH)D5 are suggestive of its promise in chemoprevention. 1α(OH)D5 has consistently been shown to be effective in inhibiting growth of cancer cells as well as preneoplastic lesions in mammary glands *in vitro*. The *in vitro* effects are manifested *in vivo* as well. In the animal carcinogenesis models, 1α(OH)D5 had reduced the incidence of tumors as well as tumor multiplicity, and increased the latency period. Yet, there were no changes in total body weight and no apparent signs of toxicity at efficacious doses. More recently, we completed preclinical toxicity studies in rats and dogs under good laboratory practices regulations that provides us with estimation of maximum tolerable dose. The concentration of 1α(OH)D5 required to achieve optimal cell regulatory effects is 100 times higher than the concentration of vitamin D_3. However, there is no hypercalcemia observed at this dose of 1α(OH)D5 to warrant concern. The mechanism of action of 1α(OH)D5 seems to involve VDR as well as cross-talk with estrogen signalling pathway. It has been shown to inhibit estrogen induced proliferation. Due to these properties, 1α(OH)D5 might prove suitable in a variety of applications. Furthermore, the differential gene expression profile clearly suggested that the effects of 1α(OH)D5 involve multiple pathways and genes, some of which have not yet been critically studied.

A scheme of possible applications of 1α(OH)D5 are presented in figure VI. From a prevention point of view, 1α(OH)D5 might be used in populations which are at high risk or to prevent or delay recurrence of breast tumors in breast cancer patients. It might also be used in conjunction with other treatments for cancer therapy. Further studies are

underway in our lab to determine if indeed 1α(OH)D5 would become available for clinical use in future.

Figure VI

ACKNOWLEDGEMENTS

Supported by RO1-CA-82316, US-DAMD-4440, and US DAMD-17-01-1-0272.

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Figure I. Structure of 1α(OH)D5 and its Ca^{++} mobilizing activity in mammals.

Figure II. Binding affinity of 1α(OH)D5 to VDR in comparison with 1α,25$(OH)_2D_3$ (Ray and coworkers, unpublished data).

Figure III. Chemopreventive efficacy of 1α(OH)D5 in inhibiting mammary alveolar (MAL) and ductal (MDL) leisions in mouse mammary gland organ culture in comparison to 1α,25$(OH)_2D_3$.

Figure IV. Down-regulation of estrogen (A) and progesterone (B) receptor transcription with vitamin D_3 and its analog in BT-474 cells as determined by RT-PCR. Lanes: 1 ⇒ control; 2 ⇒ 1α,25$(OH)_2D_3$; 3 ⇒ 1α(OH)D5.

Figure V. Down-regulation of progesterone receptor (PR) expression with 1α(OH)D5 treatment in BT-474 cells as detected by immuno-cytochemical analysis. Percentage of cells stained positive for PR were 78 % in control (A) and 46 % in treated (B) cells.

Figure VI. Potential application of 1α(OH)D5 in breast cancer prevention and therapy.

Table 1. Calcemic activity of 1α(OH)D5 in Sprague Dawley rats.

Treatment	Sample Size	Dose	Plasma Ca^{++} (mg/dL)
Vitamin D-deficient male rats		(μg/kg body weight)	
Control	8	0.0	5.4 ± 0.28
1α(OH)D5	8	0.042	6.0 ± 0.63
$1\alpha(OH)_2D_3$	8	0.042	8.1 ± 1.2 *
Vitamin D-sufficient female rats		(μg/kg diet)	
Control	15	0.0	7.0 ± 1.19
1α(OH)D5	15	25.0	7.4 ± 1.10
$1\alpha,25(OH)_2D_3$	15	12.8	8.5 ± 1.17*

* significantly different from control ($p < 0.05$).

Table 2. Growth response of various breast cancer cell lines to 1α(OH)D5 treatment.

Cell Lines	VDR Status	ER Status	PR Status	% Inhibition*	Net Effect of 1α(OH)D5
BT-474	+	+	+	50	cell cycle arrest, apoptosis
MCF-7	+	+	+	45	cell cycle arrest, apoptosis
ZR-75-1	+	+	+	30	growth inhibition
T-47D	+	+	+	30	growth inhibition
UISO-BCA-4	+	–	–	40	growth inhibition, differentiation
MDA-MB-231	–	–	–	none	none
MDA-MB-435	–	–	–	none	none

* percent growth inhibition at 1 μM 1α(OH)D5 for 72 hours, adjusted for control.

Table 3. Efficacy of 1α(OH)D5 in preventing carcinogenesis in animal models.

Tissue	Sample	Dose	Duration [d]	Tumor Incidence	Multiplicity
MNU-induced tumors in	15	0.0	17	80 %	1.6
rats	15	50 μg/kg diet	17	47 % [a]	0.8 [a]
DMBA-induced tumors	20	0.0	22	85 %	1.9
in rats	20	20 μg/kg diet	22	40 % [a]	1.3
UISO-BCA-4 xenograft	5	0.0	6	x	NA
in athymic mice [c]	5	8 ng (s.c.) [b]	6	x	NA
UISO-BCA-4 xenograft	5	0.0	6	x	NA
in athymic mice [c]	5	12.5 μg/kg diet	6	x	NA
BT-474 xenograft in	5	0.0	8.5	0.01 cm^3	NA
athymic mice [c]	5	12.5 μg/kg diet	8.5	0.125 cm^3 [a]	NA

[a] significantly different from control ($p < 0.05$).
[b] 8 ng 1α(OH)D5 subcutaneously injected thrice weekly for 60 days.
[c] results are expressed as tumor volume(cm^3).
[d] duration in weeks.

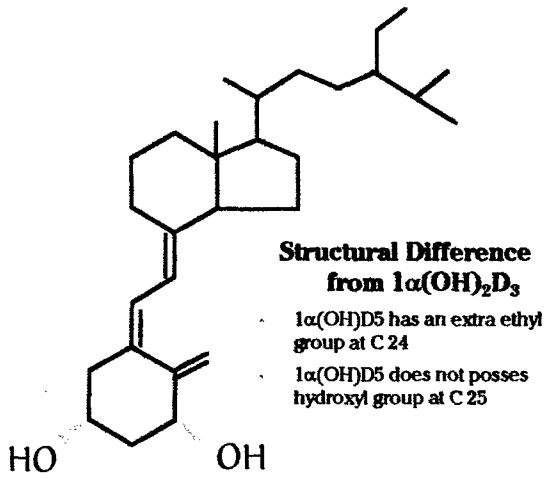
Table 4. Growth effects of 1 μM 1α(OH)D5 on normal and transformed MCF-12F breast epithelial cells.

Cell Line	Treatment	Cell Count	Cell Cycle (% G-1)	MTT Absorbance
MCF-12F	Control	47250 ± 474	68	0.045 ± 0.06
	1α(OH)D5	45820 ± 587	71	0.044 ± 0.04
$MCF\text{-}12F_{MNU}$	Control	91800 ± 120	43	0.185 ± 0.06
	1α(OH)D5	73616 ± 138 *	65	0.078 ± 0.01 *
$MCF\text{-}12F_{DMBA}$	Control	105470 ± 42.4	49	0.128 ± 0.02
	1α(OH)D5	8035 ± 91 *	67	0.075 ± 0.01 *

* significantly different from control ($p < 0.05$).

Table 5. Microarray analysis to determine effects of 1 μM 1α(OH)D5 and MNU-induced transformation on selected genes.

Comparison	Genes Upregulated	Genes Downregulated
BT-474 (control) ± 1α(OH)D5 Incyte™ Genomics Human UniGene 1 (8 K)	cytochrome P450 (vitamin D_3 24-hydroxylase), caspase 3, cadherin 18 type 2	trefoil factor 1 (pS2), progesterone receptor, trefoil factor 3, MMP-9, thymidine kinase 2 (mitochondrial), transcobalamine
MCF-12F (control) *vs* $MCF\text{-}12F_{MNU}$ Clontech Atlas™ Arrays (10 K)	TGFα, prohibitin, calpain 4, pituitary tumor transforming 1, HSP-27, thioredoxin, kearatin 6A & 6B	E2F-4, integrins, glutathione peroxidase 4, ornithine decarboxylase antizyme 1, cystatin B, tissue inhibitor of metalloproteinase 1, TCTP-1
$MCF\text{-}12F_{MNU}$ (control) ± 1α(OH)D5 Clontech Atlas™ Arrays (10 K)	glutathione peroxidase 4, ornithine decarboxylase antizyme 1, cystatin B, tissue inhibitor of metalloproteinase 1, TCTP-1, integrin β4, cadherin 3, cathepsin D	prohibitin, vimentin, MAPK-7, thioredoxin, HSP-27



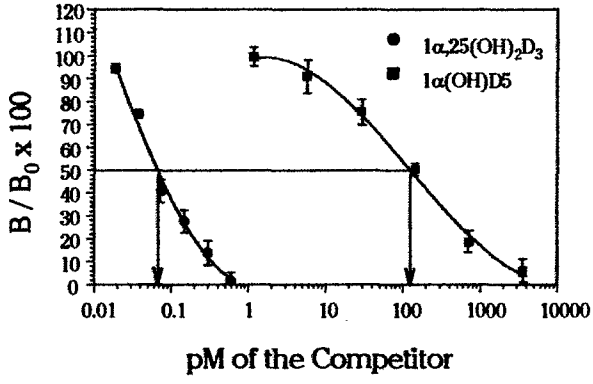
Structural Difference from $1\alpha(OH)_2D_3$

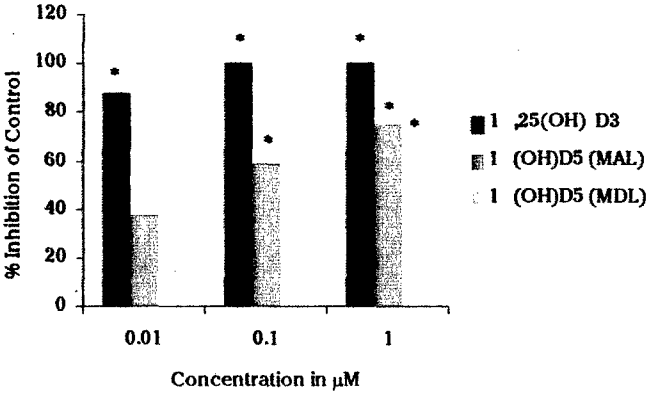
- 1α(OH)D5 has an extra ethyl group at C 24
- 1α(OH)D5 does not posses hydroxyl group at C 25

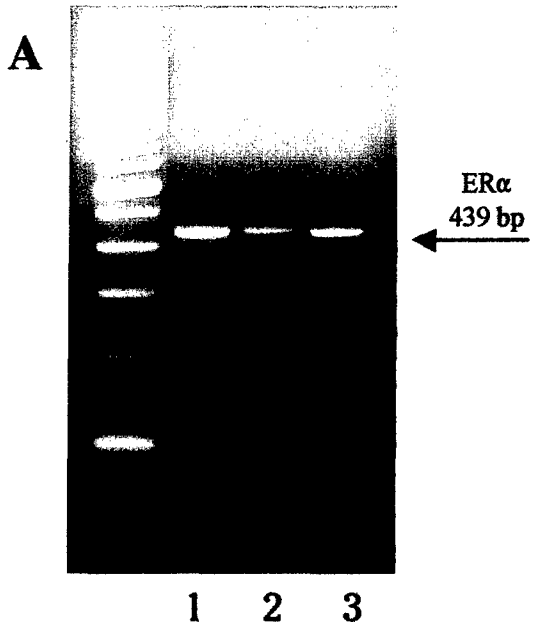
Calcemic Activity

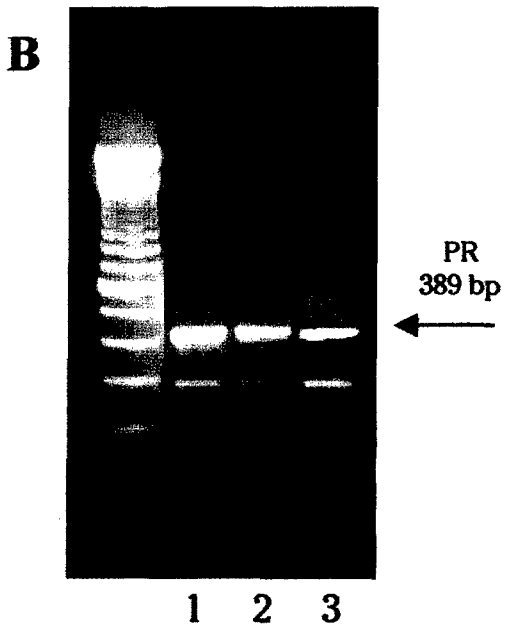
$D_3 = D_2 > D_4 > D_6 > D_5$

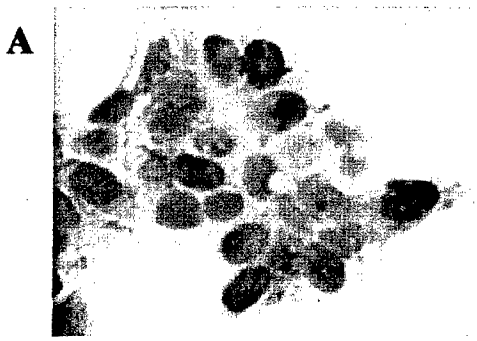
1α-hydroxy-24-ethyl-Cholecalciferol (1α[OH]D5)

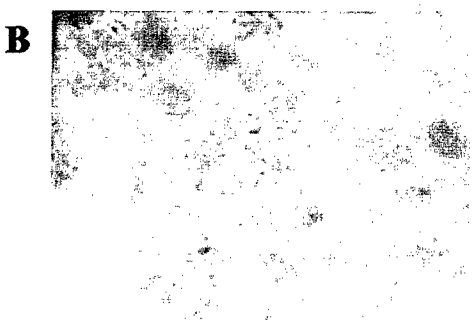












Anti-promotional
High Risk Group
delay of onset
prevention of recurrence

Preventive
General Population
non-toxic
food based

Therapeutic
Breast Cancer Patients
adjuvant therapy

1α(OH)D5

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Chemoprevention of mammary carcinogenesis by 1α-hydroxyvitamin D_5, a synthetic analog of Vitamin D

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Received 1 April 2002; received in revised form 10 August 2002; accepted 3 September 2002

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 D_5 or 1α(OH)D_5], and showed that it was tolerated by rats and mice at a much higher dose than 1α,25 dihydroxy cholecalciferol [1α,25$(OH)_2D_3$]. This property makes it a prime candidate for chemoprevention studies. In the mouse mammary gland organ culture (MMOC), 1α(OH)D_5 inhibited carcinogen-induced development of both mammary alveolar and ductal lesions. In vivo carcinogenesis study showed statistically significant reduction of tumor incidence and multiplicity in *N*-methyl-*N*-nitrosourea (MNU)-treated rats that were fed 25–50 μg 1α(OH)D_5/kg diet. There were no adverse effects on plasma calcium concentrations. In order to determine if the effect of 1α(OH)D_5 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)D_5 induced apoptosis and cell cycle G1 phase arrest in BT474 breast cancer cells without having any effects on proliferation of the MCF12F cells. In addition, in MMOC it had no growth inhibitory effects on normal epithelial cell proliferation in the absence of carcinogen. Similarly, non-tumorigenic human breast epithelial cells in explant culture did not respond to 1α(OH)D_5, whereas treatment with 1α(OH)D_5 induced cell death in the explants of cancer tissue. These results collectively indicate that 1α(OH)D_5 selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. Interestingly, the growth inhibitory effects of 1α(OH)D_5 were observed in Vitamin D receptor positive (VDR^+) breast cancer cells, but not in highly metastatic VDR^- breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1α(OH)D_5 action may be mediated, in part, by VDR.

Keywords: Vitamin D; Mammary carcinogenesis; Chemoprevention

1. 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

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doi:10.1016/S0027-5107(02)00341-X

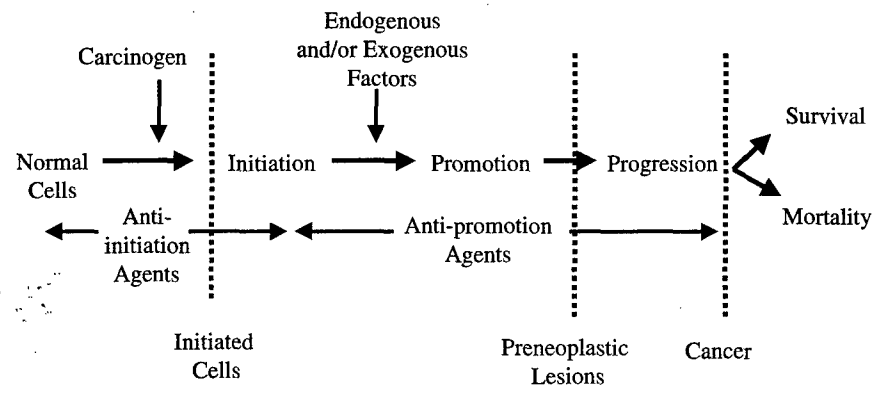

Fig. 1. Schematic diagram to show stages in mammary carcinogenesis and potential points of intervention by chemopreventive agents.

cell to a transformed cancer cell is summarized in Fig. 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 the potential to develop into malignant cancer, they may or may not form a tumor depending upon exposure to exogenous and/or endogenous factors. In the absence of growth arrest stimuli, the initiated cell can 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-initiation agents. On the other hand, chemicals that prevent further progression of initiated cells into transformed ones are termed 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. [5–7]. In this report, we have summarized the chemopreventive properties of a newly evaluated Vitamin D analog, 1-α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D_5].

It has been well established that the active metabolite of Vitamin D, 1α,25-dihydroxyvitamin D_3, [1,25$(OH)_2D_3$] 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$(OH)_2D_3$ could be of clinical significance in prevention or treatment of cancer of several target organs [10]. However, one major limitation in its clinical application is the fact that the efficacious concentrations of 1α,25$(OH)_2D_3$ are cytotoxic [11]. The effective growth inhibitory concentration of 1α,25$(OH)_2D_3$ 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 the Vitamin D molecule for the prevention and treatment of cancer. Some of these analogs have been successfully evaluated for 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 (Fig. 2): ring A, open ring B, ring CD, and the side chain. Modifications can be made at all four sites, but the alteration of the ring CD is not common due to its rigid structure. Most 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 the toxicological profile of the Vitamin D series of compounds, including D_2–D_6, had suggested that D_5 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)D_5 [17]. The structure of 1α(OH)D_5 is shown in Fig. 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic

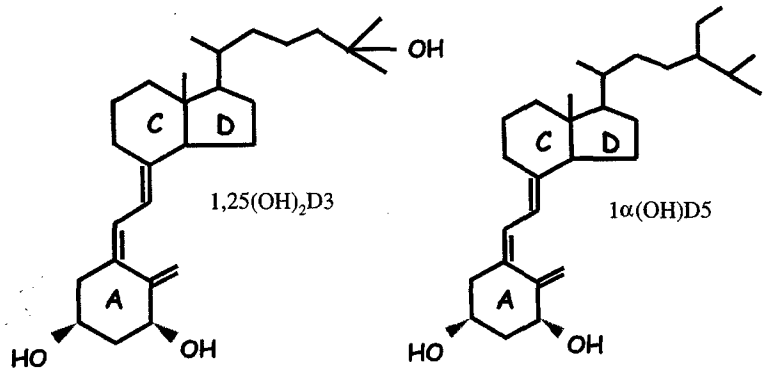

Fig. 2. Structural representation of $1,25(OH)_2D_3$ and its analog $1\alpha(OH)D_5$.

pathway involves its association with high-affinity specific Vitamin D receptor (VDR) that belongs to the steroid receptor superfamily of ligand-activated transcription factors [18–20]. 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, which translates to a 50-kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell, yielding 10–100 fmoles/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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha(OH)D_5$.

2. Materials and methods

2.1. Cell lines

We purchased from the American Type Culture Collection (ATCC), Bethesda, MD and maintained in our laboratory according to the ATCC recommendations the following cell lines: (1) the non-tumorigenic, estrogen receptor-negative (ER^-), progesterone receptor-negative (PgR^-), and low VDR breast epithelial cell line MCF12F; (2) ER^+, PgR^+, and VDR^+ breast cancer cell lines BT474 and MCF7; and (3) ER^-, PR^-, and VDR^- breast cancer cell lines MDA-MB-231 and MDA-MB-435.

2.2. Mouse mammary gland organ culture (MMOC)

The detailed procedures for culturing mammary glands from Balb/c mice have been previously reported in the literature [17,25] and outlined in Fig. 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Waymouth's MB752/1 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 h 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

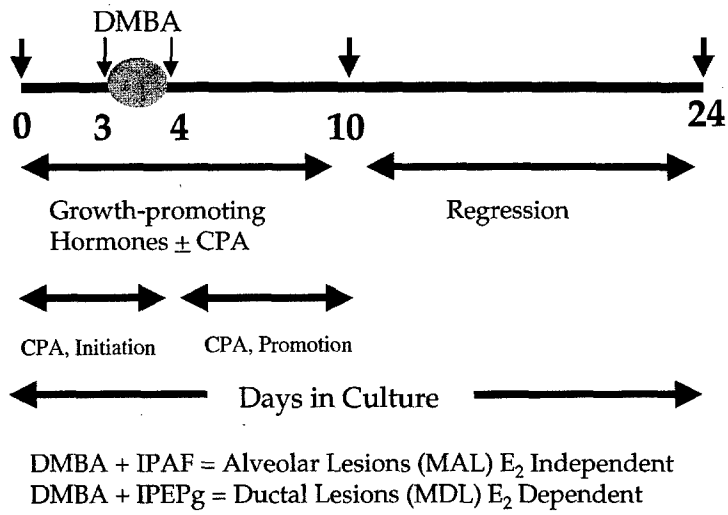

Fig. 3. Experimental design for chemoprevention in mouse mammary gland organ culture (MMOC). DMBA: 7,12-dimethylbenz(a)anthracene, CPA: chemopreventive agent, IPAF: insulin + prolactin + aldosterone + hydrocortisone, IPEPg: insulin + prolactin + estradiol + progesterone, MAL: mammary alveolar lesions,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

glands develop mammary ductal lesions (MDL) with DMBA treatment [26]. We performed a dose response study to compare the effects of $1\alpha(OH)D_5$ on MAL and MDL. Mammary lesions developed in the absence of $1\alpha(OH)D_5$ served as controls. Additionally, we determined the effects of $1\alpha(OH)D_5$ on normal mammary glands, where the glands were incubated with growth-promoting hormones and 1 μM $1\alpha(OH)D_5$ for 6 days without DMBA treatment. The glands from these MMOC experiments were fixed, stained, and analyzed for morphological characteristics and cell growth and compared with the appropriate controls.

2.3. Cell cycle analysis by flow cytometry

To determine cell cycle, we used flow cytometric analysis 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 min. This was followed by incubation with trypsin inhibitor and RNAase A. The cells were then stained with 0.04% propidium iodide solution. Approximately 10,000 cells were analyzed for DNA content using a Beckman-Coulter EPICS Elite ESP flow cytometer. Multicycle analysis software was used to determine the percentage of cells in various stages of cell cycle. Each experiment was repeated twice and student's *t*-test was used to assess differences.

2.4. Apoptosis

Programmed cell death was evaluated using acridine orange staining. Briefly, a 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 40× objective lens using a flourescein filter. Approximately 100 cells were counted on each slide to assess the proportion of cells undergoing apoptosis.

2.5. Mammary carcinogenesis

The procedure for induction of mammary adenocarcinomas by *N*-methyl-*N*-nitrosourea (MNU) in Sprague–Dawley female rats has been described in detail previously [28] and is illustrated in Fig. 4. Briefly, 100-day-old female Sprague–Dawley rats

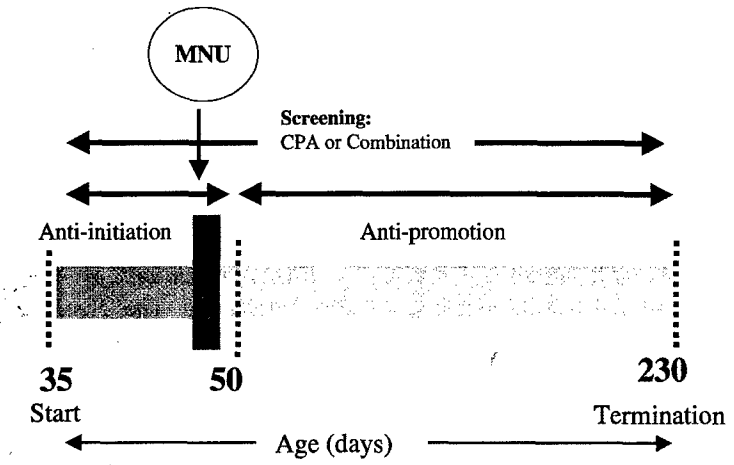

Fig. 4. Schematic diagram to show in vivo model of chemoprevention in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague–Dawley rats. CPA: chemopreventive agent.

were injected subcutaneously with 50 mg/kg MNU prepared in acidified saline. Animals received either placebo or $1\alpha(OH)D_5$ 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 $1\alpha(OH)D_5$ on the incidence, multiplicity, and latency of tumor development, and data were subjected to appropriate statistical analyses.

2.6. Effects of $1\alpha(OH)D_5$ 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 1 μM $1\alpha(OH)D_5$ were determined on these tissues by evaluating cell morphology, apoptosis, and expression of Ki 67. The effects of $1\alpha(OH)D_5$ on cell morphology and Ki 67 were compared between the normal and adjacent cancer tissue from the same patient.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Instat® 3.0 software. All MMOC as well as MNU-induced carcinogenesis data were evaluated using χ^2 analysis. Cell viability, apoptosis, and cell cycle results were assessed using two-tailed student's *t*-test with type I error set at 0.05. Serum calcium and phosphorus data were tested with student's *t*-test as well. All in vitro experiments were performed in duplicates and repeated twice.

3. Results and discussion

3.1. Synthesis and toxicity of $1\alpha(OH)D_5$

Nearly 300 analogs of $1,25(OH)_2D_3$ have been evaluated in various experimental systems in the hope of generating analogs that are more efficacious with reduced toxicity. Among the analogs evaluated, only a few have shown potent chemopreventive and therapeutic activity. These analogs include EB1089 [29], KH1060 [30], R024-5531 [31], and 22-Oxacalcitriol [32], which are relatively nontoxic at effective concentrations in experimental models. The hexafluoro analog of $1,25(OH)_2D_3$, R024-5531, has no calcemic activity, while other analogs do express dose-related calcemia [33,34]. Since it had been reported previously that Vitamin D_5 is the least toxic series of Vitamin D compounds, we synthesized $1\alpha(OH)D_5$ with the intention of testing its chemopreventive potential. The chemical synthesis of $1\alpha(OH)D_5$ 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 $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$. As reported 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 $1\alpha(OH)D_5$ for 14 days. Subsequently, serum calcium concentrations were measured. Results showed that $1,25(OH)_2D_3$ 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 $1\alpha(OH)D_5$-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 $1,25\ (OH)_2D_3$ (0.8–12.8 μg/kg diet) and $1\alpha(OH)D_5$ (6.4–50 μg/kg diet) for 2 months. Calcium concentration was increased by $1,25\ (OH)_2D_3$ treatment, while no serum calcium elevation was observed in $1\alpha(OH)D_5$-treated (25 μg/kg diet) animals (Table 1). There was no effect on the final body weight at any dose of $1\alpha(OH)D_5$ used in this study. These results indicate that $1\alpha(OH)D_5$ 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 $1\alpha(OH)D_5$ than are rats. We concluded from those studies that $1\alpha(OH)D_5$ is 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, $1\alpha(OH)D_5$ can be tolerated at a higher concentration without other toxicity outcomes.

Table 1
Effects of $1\alpha(OH)D_5$ treatment on serum calcium and phosphorous levels in Sprague–Dawley rats ($n = 10$)

Agent	Dose (μg/kg)	Serum Ca (mg/dl)	Serum P (mg/dl)[a]	BW (% gain)
None		6.3	3.6	100
$1,25(OH)_2D_3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5*	8.9	70*
$1\alpha(OH)D_5$	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

* Significantly different from control ($P < 0.05$).
[a] Significance not determined.

Chemoprevention of mammary carcinogenesis by $1\alpha(OH)D_5$: The chemopreventive properties of $1\alpha(OH)D_5$ 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 Fig. 3, the 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 $1\alpha(OH)D_5$ inhibits the development of mammary lesions in a dose-responsive manner [17]. However, it requires 10-fold higher concentration than the effective concentration of $1,25(OH)_2D_3$. The most effective dose of $1,25(OH)_2D_3$ in suppressing >60% incidence of MAL is 10^{-7} M, while $1\alpha(OH)D_5$ is equally effective at 10^{-6} M without showing cytotoxicity. We also evaluated $1\alpha(OH)D_5$ effects in the MDL model [25]. The results are summarized in Fig. 5. We found $1\alpha(OH)D_5$ to be equally effective against alveolar and ductal lesions.

Since most of the effects of Vitamin D are mediated through VDR, we determined VDR induction by $1\alpha(OH)D_5$ 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, $1\alpha(OH)D_5$ 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 $1\alpha(OH)D_5$ in an in vivo model. Prior to conducting in vivo carcinogenesis studies, a dose tolerance study was conducted in Sprague–Dawley rats. Animals were provided with increasing concentrations of $1\alpha(OH)D_5$, ranging from

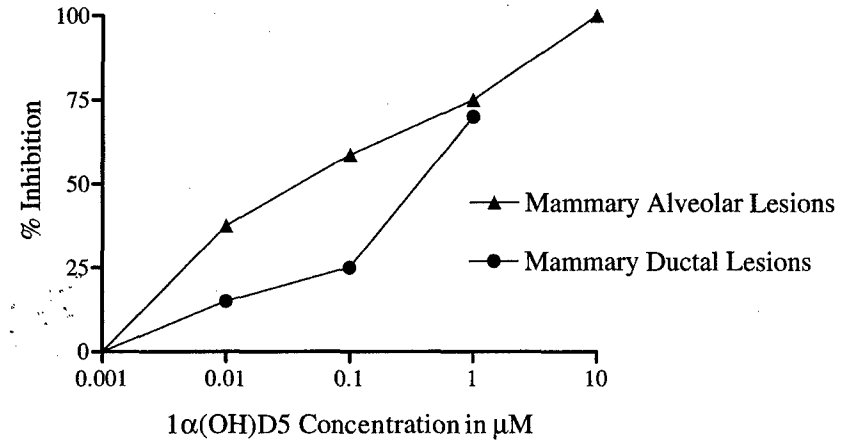

Fig. 5. Effect of $1\alpha(OH)D_5$ on mouse mammary organ culture (MMOC). The glands were incubated with 1 μM $1\alpha(OH)D_5$ for 10 days. The glands were fixed and evaluated for inhibition of preneoplastic lesions in relation to control. Fifteen glands were used per group. A difference in inhibition of greater than 60% was considered significant ($P < 0.05$, χ^2). Data shows significant inhibition of preneoplastic MAL and MDL with $1\alpha(OH)D_5$ treatment.

1 to 100 μg/kg diet for 6 weeks. The animals did not show any adverse effects at any concentration of $1\alpha(OH)D_5$, while the natural hormone was toxic at 3.5 μg/kg diet.

For the MNU-induced mammary carcinogenesis studies, animals were fed $1\alpha(OH)D_5$ at 25 and 50 μg/kg diet for 3 months. The experimental diet was given to the animals 1 week prior to the carcinogen treatment and continued until the end of the study. Results are shown in Table 2. The results indicated a dose-dependent suppression of tumor incidence by $1\alpha(OH)D_5$. This was accompanied by a reduction in tumor multiplicity and an 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 $1\alpha(OH)D_5$ to be developed as a chemopreventive and therapeutic agent.

Table 2
Chemoprevention of MNU-induced mammary carcinogenesis by $1\alpha(OH)D_5$ in rats

Treatment	Dose (μg/kg)	*n*	Incidence (%)	Multiplicity	Final BW (g)
Control	0	15	80	1.6	228
$1\alpha(OH)D_5$	25	15	53*	1.2	230
$1\alpha(OH)D_5$	50	15	47*	0.8*	226

* Significantly different from control ($P < 0.05$).

3.2. Selectivity of $1\alpha(OH)D_5$ action for transformed cells

We compared the growth effects of $1\alpha(OH)D_5$ in various steroid receptor-positive as well as negative breast epithelial cell lines. These cell lines included (1) non-tumorigenic MCF12F breast epithelial cells, (2) ER^+, PgR^+, VDR^+, BT474, and MCF7 cells, and (3) ER^-, PgR^-, and VDR^- highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ 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^+, and 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 the presence of VDR is necessary to potentiate Vitamin D's effect, it does not explain the lack of Vitamin D's effect on MCF12F cells that express low levels of VDR.

In order to examine whether $1\alpha(OH)D_5$ selectively inhibits cell proliferation in transformed cells only, we evaluated the effects of $1\alpha(OH)D_5$ on non-tumorigenic breast epithelial cells and compared them to the effects on BT474 breast cancer cells.

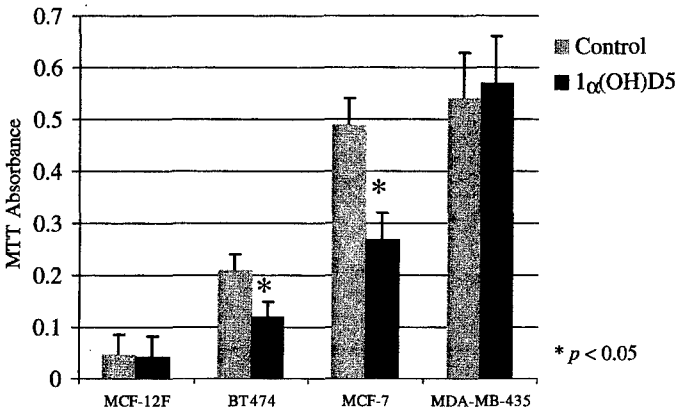

Fig. 6. Effects of $1\alpha(OH)D_5$ on viability of non-tumorigenic and cancer breast epithelial cells. Different cell lines were treated with 1 μM $1\alpha(OH)D_5$ for 2 days and incubated with MTT for 2 h. The cells were lysed and washed prior to reading absorbance at 550 nm. MTT absorbance is proportional to the number of live cells. Each experiment was repeated twice and differences between the mean were assessed using student's *t*-test.

As shown in Fig. 6, incubation of MCF12F breast epithelial cells for 6 days with $1\alpha(OH)D_5$ at 1 μM concentration did not result in suppression of cell proliferation as determined by the MTT absorbance assay. On the other hand, there was a significant inhibition of proliferation in both MCF7 and BT474 cells with $1\alpha(OH)D_5$ treatment. These results suggested that the effect of Vitamin D analog might be selective for transformed cells. The antiproliferative effects of $1\alpha(OH)D_5$ were also evident in in vivo experiments. Xenograft of ER^+, PgR^+, VDR^+, MCF7, ZR75/1, and BT474 cells or ER^-, PgR^-, VDR^+, and BCA-4 cells responded to 12.5 μg $1\alpha(OH)D_5$/kg diet and showed suppressed growth of these cells in athymic mice [35].

To confirm the selectivity of $1\alpha(OH)D_5$ for transformed breast cancer cells, we conducted three separate experiments. In the first experiment, we compared the efficacy of $1\alpha(OH)D_5$ 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 1 μM $1\alpha(OH)D_5$ for 6 days resulted in 50% growth inhibition in $MCF12F_{MNU}$ cells without having any significant effects on MCF12F growth.

In a second study using the MMOC model, the effects of $1\alpha(OH)D_5$ were determined in mammary glands. Mammary glands respond to growth-promoting hormones and develop structurally differentiated alveoli within 6 days in culture. Incubation of glands with 1 μM $1\alpha(OH)D_5$ for 6 days did not affect the growth-promoting effects of insulin, prolactin, aldosterone, hydrocortisone, estrogen, and progesterone (Fig. 7). Contrarily, $1\alpha(OH)D_5$ showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Fig. 5).

Experiments to determine the selectivity of $1\alpha(OH)D_5$ action against transformed cells were further extended to human tissues. The effects of $1\alpha(OH)D_5$ on the explants derived from normal breast tissues were compared with those 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 h in the MEME containing 5% fetal calf serum with or without $1\alpha(OH)D_5$ at 1 μM concentration. Tissue sections were histopathologically evaluated, and Ki 67 expression was determined. Results showed that the histopathology of control and $1\alpha(OH)D_5$-treated normal breast tissue was identical with no difference in apoptosis or Ki 67 expression. On the other hand, the histological sections of the cancer tissue explants showed extensive apoptosis within the tissue with

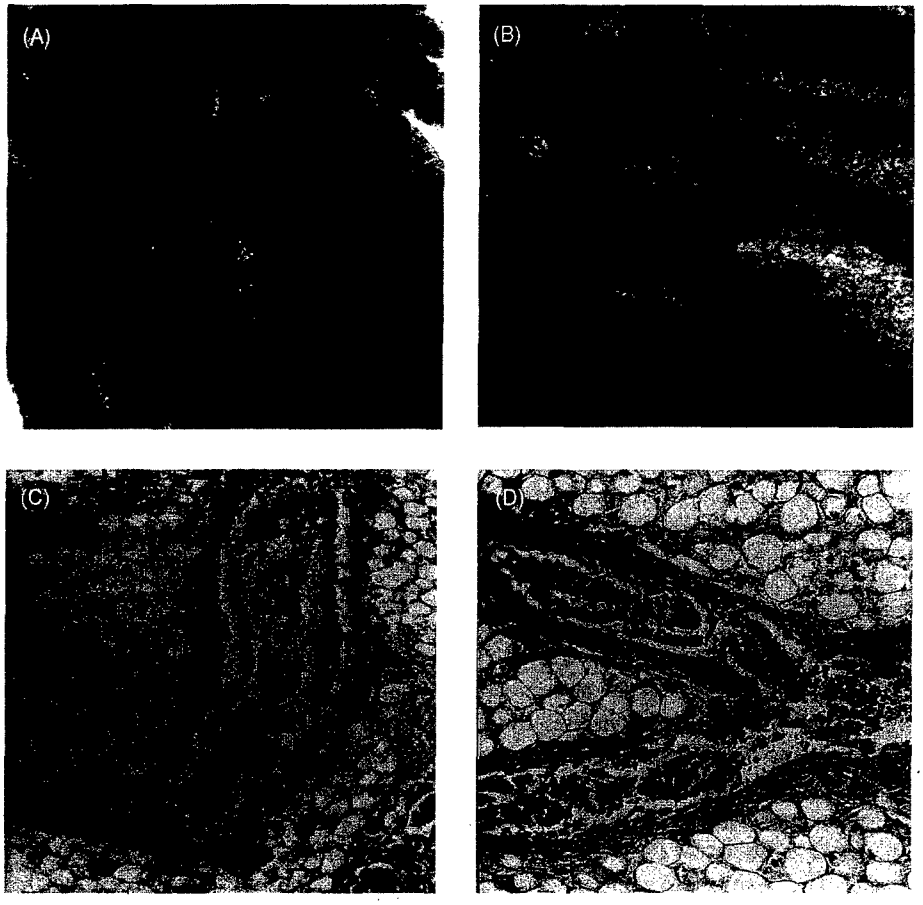

Fig. 7. The 6-day mouse mammary organ culture (MMOC) was performed without the carcinogen treatment. The data shows similar growth in both the control and 1α(OH)D_5 treated glands. (A) control; (B) 1α(OH)D_5; fixed and stained with carmine; (C) control and (D) 1α(OH)D_5, fixed, sectioned, and stained with H and E.

condensed chromatin and reduced Ki 67 expression after 72-h incubation with 1α(OH)D_5 (Mehta, unpublished data). Taken together, these results indicate that, in human breast epithelial tissues, 1α(OH)D_5 is selective for its effects on pre-cancerous or cancer cells but shows no effect on normal breast epithelial cell growth.

3.3. Mechanism of 1α(OH)D_5 action

The effects of 1α(OH)D_5 have also been evaluated in several breast cancer cell lines [37]. Although these studies do not focus directly on chemoprevention, they do provide excellent insight into the mechanism of action of 1α(OH)D_5 and its efficacy as an anti-proliferative agent. We had reported that, in ER^+, PgR^+, breast cancer cells, 1α(OH)D_5 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 [38]. 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)D_5 depended 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)D_5 on cell cycle were determined using breast cancer cells. The BT474 cells

Table 3
Effects of $1\alpha(OH)D_5$ on cell cycle phases in breast epithelial cell lines

Types	G1 (%)	S (%)	G2 (%)	G1/G2 (%)
BT474				
Control	60.7	30.5	8.8	6.9
$1,25(OH)_2D_3$	71.6*	22.1	6.3	11.4
$1\alpha(OH)D_5$	85.7*	10.3	4.0	21.4
MCF7				
Control	61.2	28.6	10.1	6.1
$1,25(OH)_2D_3$	71.9*	19.3	8.8	8.2
$1\alpha(OH)D_5$	70.0*	20.4	9.6	7.3
MDAMB435				
Control	22.8	31.3	45.9	0.5
$1,25(OH)_2D_3$	21.1	33.0	45.3	0.5
$1\alpha(OH)D_5$	21.1	23.6	55.3	0.4
MCF12F				
Control	72.4	16.2	11.4	6.4
$1,25(OH)_2D_3$	61.1*	20.2	19.0	3.2
$1\alpha(OH)D_5$	67.3*	16.2	16.5	4.1

* Significantly different from control ($P < 0.05$).

were treated with 1 μM $1\alpha(OH)D_5$ for various time points and processed for FACS analysis. Results showed that 70% of the control cells were distributed in the G1 phase, whereas treatment with $1\alpha(OH)D_5$ induced growth arrest with 84% cells in the 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 $1\alpha(OH)D_5$ treatment. Both MDA-MB-231 and MDA-MB-435 cells are devoid of steroid receptors; therefore, these cells were not expected to respond to $1\alpha(OH)D_5$ treatment. These results further confirm that the action of $1\alpha(OH)D_5$ may be mediated, in part, by VDR.

The mechanism of action of $1\alpha(OH)D_5$ was further evaluated by determining the ability of the cells to undergo apoptosis. The BT474 cells were treated with $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 72 h and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Fig. 8 shows that BT474 cells underwent apoptosis with $1\alpha(OH)D_5$ treatment as determined by acridine orange and ethidium bromide staining. The stain distinguishes live cells from those that are undergoing apoptosis. On the other hand, no apoptosis was observed in ER^-, PgR^-, VDR^+, BCA-4 cells, though there was an induction of differentiation as shown by casein, lipids, and α2 integrin expression [35].

Chemopreventive agents are being developed mostly for people who do not yet have disease but are at high risk of developing cancer. Here, we show that the Vitamin D analog might be selective for transformed cells. The population at high risk of developing cancer is assumed to be initiated for carcinogenesis and, as we have shown, initiated cells respond well to $1\alpha(OH)D_5$. In addition, we also showed here that $1\alpha(OH)D_5$ is effective against steroid-responsive cancer cells. These results suggest that $1\alpha(OH)D_5$ can be considered as a possible chemopreventive and therapeutic agent. Moreover, if given in combination with other agents, it may provide synergistic protection.

It is unclear as to where chemoprevention ends and chemotherapy begins. However, the clear principle

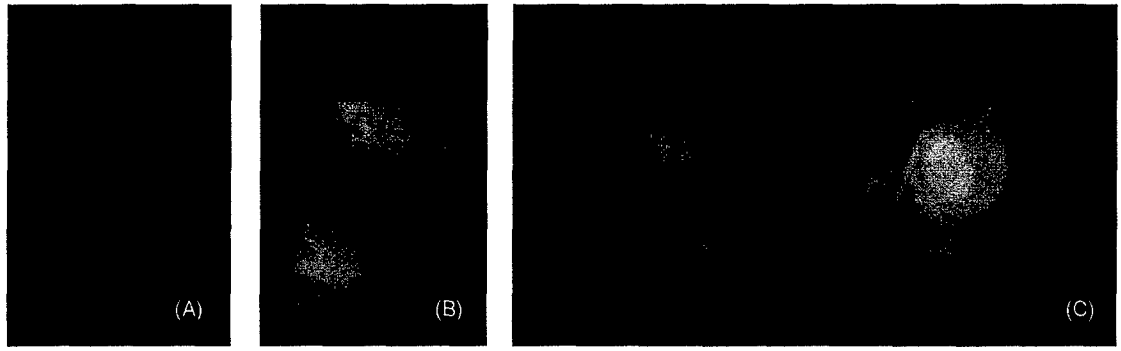

Fig. 8. Induction of apoptosis in BT474 cells by 1 μM $1\alpha(OH)D_5$, as determined by acridine orange and ethidium bromide staining. (A) control; (B) $1,25(OH)_2D_3$ (0.1 μM); (C) $1\alpha(OH)D_5$ (1 μM).

and prerequisite of chemoprevention is that the agent should not have any adverse effects. The lack of toxicity of $1\alpha(OH)D_5$ at an effective concentration may provide a rationale for its role in chemoprevention and therapy.

In summary, we have described here the chemopreventive properties of a relatively new non-toxic analog of Vitamin D, $1\alpha(OH)D_5$, against mammary carcinogenesis models. In addition, our results suggest that $1\alpha(OH)D_5$ may be active selectively against transformed cells without showing adverse effects on normal breast epithelial cells.

Acknowledgements

This work was supported by the Public Health Service Grant R01-CA82316, US Army DAMD 17-97-17263 and DAMD 17-01-10272.

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