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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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Abstract
It is now well established that the active metabolite of vitamin D₃, 1α,25(OH)₂D₃, 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 D₃ analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models. We have previously reported an analog of vitamin D₃, l-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. Moreover, D5 showed a selective growth inhibitory action on transformed MCF-12F cells while no growth inhibitory effect was observed on normal MCF-12F cells. Similarly, D5 induced growth arrest and/or differentiation in hormone responsive breast cancer BT-474 and MCF-7 cells. D5 induced its growth inhibitory effect by modulating expression of various cell cycle related proteins. Among the major cell cycle check points that were up regulated by D5, were CDKIs p21 and p27 in BT-474 cells. In the MCF-12F cell model, D5 reduced expression of epidermal growth factor receptor, over expression of which is associated with hormone resistance. There was no effect of D5 on EGFR expression in the breast cancer BT-474 and MCF-7 cells. Thus, the effect of D5 appears to be cell model specific. On the other hand, in the BT-474 cell model, D5 down regulated the transcription and expression of estrogen receptor alpha (ERα). Down regulation of ERα was followed by decreased expression of progesterone receptor and the trefoil factor pS2 suggesting anti-estrogenic action of D5. The gene modulating effects of D5 seemed to be mediated via vitamin D receptor (VDR) as D5-induced up regulation of VDR preceded its effects on gene expression and growth inhibition. In conclusion, D5 appears to be a candidate for breast cancer prevention and therapy and requires further development as a chemopreventive and therapeutic agent. (Supported by US Army Research Materiel Command under DAMD17-01-1-0272).
TABLE OF CONTENTS

A. Cover .................................................................................................................................
B. Standard Form 298 ............................................................................................................
C. Introduction ........................................................................................................................
D. Specific Aims and Statement of Work ..............................................................................
E. Study Design and Methods ............................................................................................
F. Results ..............................................................................................................................
G. Key Research Accomplishments ....................................................................................
H. Reportable Outcomes ........................................................................................................
I. Conclusion and Studies in Progress ..................................................................................
J. References ........................................................................................................................

LIST OF FIGURES

1. Project time-line for the entire study ..............................................................................
2. Invasive potential of MCF-12F_{MNU} transformed cells ............................................
3. Normal and transformed MCF-12F cell viability after 2-day D5 treatment ..................
4. Effect of D5 treatment on BT-474 cell growth ..............................................................
5. Detection of apoptosis by TUNEL assay in D5 treated BT-474 cells ............................
6. D5 effect on cell cycle-related protein expression in BT-474 cells .................................
7. Steady state levels of VDR mRNA after D5 treatment in BT-474 cells .........................
8. Steady state levels of ER mRNA after D5 treatment in BT-474 cells ............................
9. Steady state levels of PR mRNA after D5 treatment in BT-474 cells ............................
10. Expression of PR upon treatment with D5 in BT-474 cells ...........................................

LIST OF TABLES

I. Micro-array comparison of MCF-12F with MCF-12F_{MNU} and MCF-12F_{MNU} control with D5-treated cells .......................................................................................................................... 4
II. Micro-array comparison of BT-474 control with D5-treated cells ................................ 5
III. Cell cycle analysis by flow cytometry ........................................................................... 6
INTRODUCTION

Breast cancer is the second major cause of cancer deaths among women. The active form of vitamin D (1,25(OH)₂D₃ or VD₃) has been well recognized as an effective growth suppressing agent for leukemia and breast, colon, and prostate cancers. Several in vitro studies support the role of VD₃ as an antiproliferator and inducer of differentiation in breast cancer cells. 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 VD₃. Our lab has been studying an analog of vitamin D, 1α-hydroxy-24-ethyl cholecalciferol (D5), for the past four years. This analog has shown antiproliferative effects in carcinogen-transformed mouse mammary gland organ culture (MMOC) and steroid receptor positive breast cancer cells in vitro with little or no calcemic activity in vivo. Therefore, it is a good candidate for further investigations.

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

Previously, we have tested growth effects of D5 on normal human breast epithelial cells (MCF-12F), and observed no growth inhibition at 0.1 μM concentration. We transformed these normal cells to pre-neoplastic stage by using two different types of carcinogens. After establishment of transformed cell lines, we compared and studied the effects of D5 on cell growth, gene expression patterns, and cell cycle progression in normal and transformed cells. Furthermore, we conducted some preliminary studies to determine possible interaction of D5 with estrogen and estrogen receptor in breast cancer cells. 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 and cancer cells as compared to normal cells?
- Does D5 have an inhibitory action on the expression of cell invasion and proliferation markers and cell cycle related proteins?
- Does D5 interact with the estrogen signaling pathway in the cancerous mammary epithelial cells?
SPECIFIC AIMS AND STATEMENT OF WORK

The overall objective of this study was to understand the mechanism of action of D5 for its use in breast cancer prevention and therapy. Specific aims included:

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

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

Task 3: Determine whether D5 modulates expression of cell cycle check points in breast cancer cells.

Task 4: Determine whether D5 regulates the expression of vitamin D receptor (VDR), estrogen receptor (ER), and estrogen inducible genes in breast cancer cells.

STUDY DESIGN AND METHODS

We maintained the MCF-12F cells according to the ATCC instructions in DMEM: Ham’s F12 mixture (1:1) with added insulin, epidermal growth factor, cholera toxin, and hydrocortisone. This cell line had been established with long-term culture in low Ca++ media. Therefore, we use 5% chelalex-treated horse serum in culture medium. We performed transformation of MCF-12F cells with two different carcinogens, DMBA that needs to be metabolized, and MNU, which is a direct acting carcinogen. Briefly, MCF-12F cells were grown to subconfluency in a tissue culture dish. Cells were incubated with DMBA (2 μg/mL) for 24 hours followed by another 24 hour incubation with fresh DMBA in the media, which caused extensive cell death. Surviving cells were washed with PBS and allowed to grow in fresh media without DMBA. For MNU-induced transformation, the carcinogen was dissolved in acidified saline (pH 5.3) and used within 20 minutes of preparation. Cells were exposed to MNU (25 μg/mL) twice daily for two days and later allowed to remain in fresh media. Treated cells underwent extensive cell death in serum starved media and the surviving cells were then plated in fresh media. The cell transformation efficiency was tested by soft agar colony formation and tumor incidence in athymic mice. The resulting cell lines were designated MCF-12F_{DMBA} and MCF-12F_{MNU}.

The growth rate was compared between the normal and transformed cell lines using Coulter cell counter and MTT absorbance assay. Cell invasiveness was determined by using Matrigel® coated Boyden chambers. MCF12F cells were plated on the membranes and percentage of cells migrating through the Matrigel® were counted. 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. Similar experiments were conducted with breast cancer cells, BT-474 and MCF-7 to determine effects of D5 on cancer cell growth.

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. We studied the expression of cell cycle markers, such as cyclins and cyclin-dependent kinases, and other proteins involved in cell cycle regulation using western blots. Western blots, and RT-PCR were used to determine VDR and ER expression and transcription, respectively, in breast cancer BT-474 cells. Appropriate statistical analysis were performed using Graphpad® Instat software and data was plotted using MS Excel 2000.

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. In order to determine whether D5 inhibits growth of transformed cells, we transformed the MCF-12F cell line using the carcinogens DMBA and MNU. After the transformation of MCF-12F cells into MCF-12F_{DMBA} and MCF-12F_{MNU}, we characterized and compared their growth rate with that of the parent cell line. The transformed cells were 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.

Interestingly, the transformed cells showed response to D5 treatment while the parent cell line MCF-12F did not respond to D5 action. Figure 3 shows the normal and transformed MCF-12F cells viability with D5 treatment. The growth of transformed cells were significantly reduced by D5 treatment, but no significant effect was observed on MCF-12F cells.

Figure 2: Invasive potential of MCF-12F_{MNU} transformed cells.

![Image of invasive potential of MCF-12F_{MNU} cells]

Figure 3: Normal and transformed MCF-12F cell viability after 2-day D5 treatment.

![Image of MTT absorbance graph]
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 while 149 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 I lists selected genes that were altered by D5 treatment.

Table I. Micro-array Comparison of MCF-12F with MCF-12F_{MNU} and MCF-12F_{MNU} Control with D5 Treated.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>MCF-12F_{MNU}</th>
<th>MCF-12F_{MNU} (D5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Shock Protein 27 kDa</td>
<td>2.7</td>
<td>-4.0</td>
</tr>
<tr>
<td>Prohibitin</td>
<td>4.1</td>
<td>-2.4</td>
</tr>
<tr>
<td>Glutathione Peroxidase 4</td>
<td>-2.7</td>
<td>3.0</td>
</tr>
<tr>
<td>Ornithine Decarboxylase Antizyme</td>
<td>-2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Cystatin B (stefan B)</td>
<td>-3.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Tumor Protein 1 (TCTP1)</td>
<td>-17.8</td>
<td>16.1</td>
</tr>
<tr>
<td>Rho GDP Dissociation Inhibitor α</td>
<td>-6.5</td>
<td>5.9</td>
</tr>
<tr>
<td>BCL2-like 1</td>
<td>-2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Tissue Inhibitor of Metalloproteinase 1</td>
<td>-3.1</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Similar to the transformed breast epithelial cells, the breast cancer BT-474 cells and MCF-7 cells showed growth inhibition upon D5 treatment (figure 4). However, the hormone resistant highly metastatic breast cancer cells, MDA-MB-231 and MDA-MB-435, did not show growth inhibition after D5 treatment. Therefore, it is likely that D5 action is selective for hormone responsive breast cancer and transformed breast epithelial cells that resemble the pre-neoplastic stage of cancer.

Figure 4: Effect of D5 treatment on BT-474 cell growth.
In order to identify gene targets of D5 in breast cancer BT-474 cells, we used Human UniGene 10K arrays. The differential gene expression profiles were determined for BT-474 cells with or without D5 treatment. The major differences were found for the estrogen inducible genes such as progesterone receptor (PR) and trefoil factor (pS2), which were significantly down regulated with D5 treatment. A list of some important genes altered by D5 in BT-474 cells is given in table II.

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

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

The cell cycle analysis for D5 treated normal and transformed MCF-12F cells as well as breast cancer BT-474 and MCF-7 cells were conducted. The results showed that D5 treatment of cells caused G-1 phase arrest in transformed MCF-12F, MCF-7, and BT-474 cells, but did not significantly alter MCF-12F cell cycle progression. The results are summarized in table III.

To determine if the cell cycle arrest would be followed by apoptosis we performed the TUNEL assay for DNA strand breaks using Intergen Apoptag® kit. Figure 5 shows the apoptotic brown stained nuclei of transformed BT-474 cells that were treated with D5.

To further explore the G-1 phase arresting action of D5 in BT-474 cells, we conducted western blot analysis to determine the expression profile of cell cycle related proteins upon D5 treatment. Our results indicated that cyclin dependent kinase 2 (CDK-2) and cyclins A and D1, which play a role in transition of cell cycle from G-1 to S phase were modestly down-regulated in D5 treated BT-474 cells. While, the cyclin dependent kinase inhibitors (CDKI) p21^{waf1} and p27^{kip1} were up-regulated upon D5 treatment in BT-474 cells, explaining the G-1 phase arrest (figure 6). Furthermore, an S-phase related transcription factor E2F-1 was also modestly down-regulated by D5 treatment.
### Table III. Cell Cycle Analysis by Flow Cytometry.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>% Cells in G-1 phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-474</td>
<td>Control</td>
<td>60.7</td>
</tr>
<tr>
<td></td>
<td>D5 (1 μM)</td>
<td>85.3</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Control</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>D5 (1 μM)</td>
<td>71.0</td>
</tr>
<tr>
<td>MCF-12F</td>
<td>Control</td>
<td>62.9</td>
</tr>
<tr>
<td></td>
<td>D5 (1 μM)</td>
<td>65.2</td>
</tr>
<tr>
<td>MCF-12FDMBA</td>
<td>Control</td>
<td>45.1</td>
</tr>
<tr>
<td></td>
<td>D5 (1 μM)</td>
<td>65.7</td>
</tr>
<tr>
<td>MCF-12FMNU</td>
<td>Control</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td>D5 (1 μM)</td>
<td>59.3</td>
</tr>
</tbody>
</table>

* p value < 0.05

---

**Figure 5:** Detection of apoptosis by TUNEL assay in D5 treated BT-474 cells.

**Figure 6:** D5 effect on cell cycle-related protein expression in BT-474 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\(_{\text{MNU}}\) cells that induced response to D5 treatment. Interestingly, there was very modest induction of VDR in MCF-12F\(_{\text{MNU}}\) cells, while MCF-12F\(_{\text{DMBA}}\) 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 ER\(\alpha\), but show moderate expression of epidermal growth factor receptor (EGFR). MCF-12F\(_{\text{DMBA}}\) showed high levels of EGFR that was unaltered by D5 treatment, while MCF-12F\(_{\text{MNU}}\) expressed moderate levels of EGFR that was down-regulated in D5 treated cells. Vitamin D\(_3\) has been shown to down-regulate EGFR in colon and other cancer cells, which was followed by growth suppression of these cell lines\(^7\). Our reports indicated that the D5-induced decreased expression of EGFR in MCF-12F\(_{\text{MNU}}\) cells preceded the cell cycle arrest of these cells in the G-1 phase.

As mentioned earlier that the D5 effect seemed to be dependent upon target gene modulation via its receptor VDR. Therefore, we determined the regulation of VDR upon D5 treatment in the BT-474 cells. Our results indicated that VDR mRNA was up-regulated within 4 hours of D5 treatment and reached a peak at about 6 hours of treatment (figure 7). The induction of VDR was diminished upon simultaneous treatment with RNA synthesis inhibitor, actinomycin D, suggesting that D5 induced \textit{de novo} synthesis of VDR in BT-474 cells. Similar results were obtained in protein expression studies of VDR using western blot analysis on cell extracts from BT-474 control and treated cells.

![Figure 7: Steady state levels of VDR mRNA after D5 treatment in BT-474 cells.](image)

We had tested several breast cancer cell lines for D5 response\(^8\) and it appeared that D5 mainly inhibited the growth of estrogen-responsive breast cancer cell lines, such as BT-474 and MCF-7. Therefore, we decided to use BT-474 to study interaction of D5 with estrogen signalling. To investigate whether D5 treatment would effect expression of estrogen receptor alpha (ER\(\alpha\)), we performed RT-PCR in D5 treated BT-474 cells. The ER\(\alpha\) transcription decreased in response to D5 treatment (figure 8). The protein expression as assessed by western blot analysis showed similar decreases in ER\(\alpha\) upon D5 treatment in BT-474 cells.
Likewise, the expression of estrogen-inducible genes progesterone receptor (PR) and trefoil factor I (pS2) showed down-regulation upon D5 treatment, confirming the anti-estrogenic activity of D5 in BT-474 cells. Figure 9 shows the levels of PR mRNA from BT-474 cells upon D5 treatment as determined by RT-PCR, while figure 10 shows the relative PR expression as assessed by immunocytochemistry.

Figure 8: Steady state levels of ER mRNA after D5 treatment in BT-474 cells.

Figure 9: Steady state levels of PR mRNA after D5 treatment in BT-474 cells.

Figure 10: Expression of PR upon treatment with D5 in BT-474 cells.
KEY RESEARCH ACCOMPLISHMENTS

♦ Transformation of non-tumorigenic MCF-12F cells to establish MCF-12F<sub>DMBA</sub> and MCF-12F<sub>MNU</sub> 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<sub>MNU</sub> cells.
♦ Micro-array results indicating that D5 treatment restored transcription of some genes, which were altered by transformation.
♦ Down regulation of estrogen-inducible genes with D5 treatment in breast cancer cells (BT-474 and MCF-7).

REPORTABLE OUTCOME


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

Cell line development: MCF-12F<sub>DMBA</sub> and MCF-12F<sub>MNU</sub>

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

CONCLUSIONS AND STUDIES IN PROGRESS

In conclusion our studies indicate a selective growth inhibitory action of D5 on transformed cells, but not on normal-like MCF-12F cells. Additionally, our studies indicate down regulation of
estrogen inducible genes with D5 treatment. This selective action of D5 combined with weak anti-estrogenic activity can be exploited in both preventive and therapeutic measures for breast cancer. Moreover, D5 modulates expression of cell cycle proteins in breast cancer cells and arrests the cells in G1 phase of the cycle.

REFERENCES


