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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We identified a novel vitamin D analog, 1α-hydroxy-24 ethyl vitamin D_5 (1α(OH)D_5) that showed potent growth nhibitory and cell-differentiating actions in breast cancer cells. Based on our findings in vitro and in vivo, we hypothesized hat 1α(OH)D_5 (D5), when administered to women with breast cancer, will induce differentiation of dedifferentiated cells nd thereby prevent progression of malignancy. 1999-2000, we completed preclinical studies in rats, showing that D5 has no erious toxicity; a hypercalcemic effect was observed at high dose, which was reversible. In vitro study in tissues obtained rom patients show that D5 has no effect on normal breast epithelial cells but induces apoptosis in breast cancer and showed poptotic effect in fibroadenomas. We completed 5 steps in the synthesis of D5 in preparation for phase I clinical study. 000-2001, under GMP, we completed preclinical toxicity studies in dogs and completed synthesis of 1α(OH)D_5. In vitro tudies in clinical specimens suggest that D5 has no effect on normal breast tissues; it inhibits cell proliferation in tumor ells. D5 or its active metabolite possibly interacts with estrogen receptor. 2001-2002, we studied the in vitro effect of D5 n malignant and nonmalignant tissues obtained from breast cancer patients. D5 had no effect on cell proliferation, cell eath, or differentiation markers (casein) in nonmalignant breast tissues (epithelial cells). D5 induced cell death in ibroadenomas. In malignant tumors, D5 induced apoptosis (20). We have completed all prerequisites and are finalizing an ND application and planning a clinical phase I trial. Thus, a one-year no-cost extension is requested.

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Introduction

Vitamin D and its analogs have shown potential chemopreventive and chemotherapeutic effects on various malignant tumors (1-14). The active metabolite of vitamin D3, 1,25(OH)2D3, has been shown conclusively to induce differentiation in vitro in a variety of cancer cells, including breast cancer cells (12-14). 1,25(OH)2D3 is hypercalcemic, and thus its use as a preventive and therapeutic agent is limited. Although a number of vitamin D analogs are synthesized, only limited vitamin D-related compounds have reached clinical trial. Recently, we identified a vitamin D analog that showed potent growth inhibitory and cell-differentiating action in breast cancer cells. The effects of $1\alpha(OH)D_5$ were extensively investigated in vitro and in vivo. We aim to pilot $1\alpha(OH)D_5$ from an experimental laboratory model to the clinical setting. The effects of $1\alpha(OH)D_5$ were investigated extensively in in vitro and in vivo experimental models, and some pronounce effects of $1\alpha(OH)D_5$ are summarized below.

- $1\alpha(OH)D_5$ has chemopreventive action in mouse organ culture model (15).
- $1\alpha(OH)D_5$ has chemopreventive action on DMBA-induced mammary tumors in rats (16).
- $1\alpha(OH)D_5$ has both growth inhibitory and cell-differentiating actions in human breast carcinoma cells (17,18).
- $1\alpha(OH)D_5$ supplemented in the diet inhibits the in vivo growth of human breast carcinoma transplanted in athymic mice (18).
- $1\alpha(OH)D_5$ is metabolized into two major metabolites (1,24 and 1,25 vitamin D5) in human breast tumors and nonmalignant breast tissues.
- During the current grant funding period, we have completed preclinical toxicity studies in two different species. Studies were performed in male and female rats and dogs under GLP. Adult male and female rats/beagle dogs were given 1-10 μg/kg body weight $1\alpha(OH)D_5$ by gavage for 28 consecutive days. $1\alpha(OH)D_5$ in rats showed no serious toxic effect. No animals died during the course of study, and no adverse treatment-related clinical signs of toxicity were observed. Increased serum calcium levels were observed in both sexes at the high dose level and in females at mid-dose levels. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at mid- and high-dose levels, and in females at all doses (19).
- The effect of $1\alpha(OH)D_5$ was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group (19).
- Under the current contract, during the last funding year, we studied the in vitro effect of $1\alpha(OH)D_5$ on malignant and nonmalignant tissues obtained from breast cancer patients at the time of surgery. $1\alpha(OH)D_5$ had no effect on cell proliferation, cell death, or differentiation markers (casein) in nonmalignant breast tissues (epithelial cells). $1\alpha(OH)D_5$ induced cell death in fibroadenomas. In malignant tumors, $1\alpha(OH)D_5$ induced apoptosis (20). Preclinical toxicity studies in dogs and rats suggested that the compound is well tolerated and causes no serious toxicity. We have designed a clinical protocol for phase I clinical studies in breast cancer patients, the protocol is approved by both UIC IRB committee LGH IRB committee,

and the informed consent form is approved. An IND application is being finalized and will be submitted to FDA.

Hypothesis proposed

We hypothesize that (1) $1\alpha(OH)D_5$ administered to women with breast cancer will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy, and (2) in women with premalignant lesions, $1\alpha(OH)D_5$ will prevent dedifferentiation and thus prevent induction and/or development of breast cancer.

Technical Objectives proposed

The specific objectives of the proposed study are to:

1. Establish and evaluate biomarkers predicting $1\alpha(OH)D_5$ response in malignant breast cancer and DCIS (Ductal Carcinoma in Situ).
2. Study the molecular mechanism by which $1\alpha(OH)D_5$ induces differentiation/inhibits proliferation of breast cancer cells.
3. Perform (according to FDA requirement) preclinical toxicity and pharmacokinetic studies of $1\alpha(OH)D_5$.
4. Initiate a phase I/II trial in advanced breast cancer patients. (During this trial, we will also obtain data on the metabolism of $1\alpha(OH)D_5$ in humans.)

Successful completion of the proposed study will identify a new chemotherapeutic and possibly chemopreventive agent in breast cancer.

Results

Effects of $1\alpha(OH)D_5$ in normal and malignant breast tissues

For this specific aim, we accrued several malignant breast tissues (n= 13), nonmalignant breast tissues (n=24), and fibroadenomas (n=2) from women with confirmed diagnosis of the disease. Paired malignant and nonmalignant breast tissues (n=10) were obtained from women undergoing mastectomy for confirmed diagnosis of breast cancer. The effects of $1\alpha(OH)D_5$ were determined *in vitro* in explant culture of these tissues.

For *in vitro* experiments, fresh tissues received from the OR were minced into small pieces (~2-3 mm in size). A small piece was fixed immediately in 10% buffered formalin for histopathological evaluation, and the remaining tissues was processed for culture experiments and/or *in vivo* experiments. We confirmed our previous findings for experimental conditions. All tissues were incubated in an atmosphere of 95% O_2 and 5% CO_2. We incubated the tumor/tissue explants in basal culture medium at 37°C alone, or in the medium containing varying concentrations of $1\alpha(OH)D_5$ (0.1-1 μM) or $1\alpha,25(OH)_2D_3$(0.1 μM) for 0-72 hours. At the end of incubation, the tissues were fixed in 10% buffered formalin and processed for histopathological or immunohistological examination of various biomarkers (Ki-67, VDR, casein, and alpha2 integrin expression).

Effect of $1\alpha(OH)D_5$ on nonmalignant breast tissue

Our results in the current studies clearly show that human breast tissues, both malignant and nonmalignant, preserve the original histological features when incubated in culture medium

containing dextran-coated charcoal stripped serum. Histopathology of the normal breast tissue showed that alveolar and ductal morphology is preserved up to 72 hours. When normal breast tissues were incubated in 1,25(OH)2D_3 or 1α(OH)D_5, no histological changes were observed in ductal and alveolar structures. In normal tissue, no significant effect of 1α(OH)D_5 or vitamin D3 was observed. In all the tissues, viable breast epithelial structures are observed. Occasionally, the presence of cells with pyknotic nuclei was observed in both control and treated cells.

To further confirm the viability of the cells, we examined the immunoreactivity to Ki-67 antibody, a marker for cell proliferation. Originally, in breast tissue obtained from women (0 time), we observed 4-6 cells/each alveolar section undergoing proliferation, as evident from the immunoreactivity to Ki-67. This proliferation rate was maintained throughout the course of study in all control and treated tissues.

We also examined expression of intracellular expression of casein in nonmalignant tissues incubated in vitro in the presence/absence of vitamin D analog. In original tissue (0 time), no detectable expression of casein was observed immunohistochemically. Similarly, in tissues incubated for 24-72 hours in the control medium, no detectable casein immunoreactivity was observed. Enhanced immunoreactivity to casein antibody was observed in tissues incubated with 1α(OH)D_5 for 72 hours.

In nonmalignant breast, all alveolar and ductal lesions showed distinct immunostaining for VDR localized in the nucleus. No change in VDR expression was observed in control or treated tissues during the course of study.

In summary, our results in nonmalignant breast tissues show that nonmalignant breast tissues cultured in vitro maintained the alveolar and structural integrity up to 72 hours; cells are viable and proliferating. VDR is present in both alveolar and ductal cells, and it is maintained in the current culture conditions used. 1α(OH)D_5 had no effect on the cellular morphology or proliferation on breast epithelial cells, but it probably increases the expression of casein; however, these results need to be confirmed in a large number of specimens.

Effect of 1α(OH)D_5 on fibroadenoma

Fibroadenomas maintained in culture not only showed viability of the individual cells, but also preserved the essential architecture of the alveolar, ductal, and stromal components. Only occasionally, cells containing pyknotic nuclei were seen. However, when 1α(OH)D_5 was added to the medium 72 hours later, a proportion of the alveoli lost their epithelial components. The individual cells showed karyolysis and nuclear pyknosis with patchy areas of degeneration in the stromal components. Similar to the normal breast tissue, Ki-67 immunoreactivity did not show any changes after incubation with 1α(OH)D_5. In contrast, VDR expression appears to be enhanced by at least 60% in fibroadenomas grown in the medium containing 1α(OH)D_5.

We examined the effect of 1α(OH)D_5 on fibroadenoma of the breast obtained from women with confirmed diagnosis of the disease. Fibroadenoma tissues were viable when maintained in culture for 72 hours. At 72 hours in tissues incubated in control medium, we observed the presence of normal alveolar and ductal structures, and stromal elements. Breast alveolar structures consisted of distinct basement membrane; epithelial cells surrounded the lumen. All alveolar and ductal cells showed distinct nucleus and abundant cytoplasm. Occasionally, the presence of cells with pyknotic nucleus was observed. In tissues treated with

$1\alpha(OH)D_5$ in vitro for 72 hours, many of the alveolar structures appeared to be normal; however, some lost their epithelial components. Many epithelial cells showed nuclear pyknosis or karyolysis. In the areas with degenerated alveolar structures, stromal components also showed necrotic changes.

We further examined Ki-67 immunoreactivity in control and $1\alpha(OH)D_5$-treated tissues. In control tissue, numerous alveolar epithelial cells and stromal cells showed Ki-67 immunoreactivity. In $1\alpha(OH)D_5$-treated tissue, immunoreactivity to Ki-67 was similar to that of control in areas showing no degenerative changes.

In $1\alpha(OH)D_5$-treated tissue, only those areas that showed histopathology similar to that of control tissues showed distinct nuclear localization of VDR. In general, in control tissue, 30-40% of breast epithelial cells showed VDR expression, whereas in treated tissue VDR expression was observed in 90-95% of cells.

Effect of $1\alpha(OH)D_5$ on human breast carcinoma.

We examined the effect of $1\alpha(OH)D_5$ in human breast carcinoma tissues incubated in vitro in control and 1 μM $1\alpha(OH)D_5$-containing medium. In most of the malignant breast tumors studied, the original histopathological features were preserved up to 48 hours when tissues were incubated in the control medium. Very few cells in this control tissue showed apoptotic or pyknotic changes. In contrast, cells incubated for 48 hours in medium containing 1 μM $1\alpha(OH)D_5$ contained apoptotic cells. In addition, many cells at various stages of apoptotic death were observed. We are currently studying Ki-67, VDR, and casein expression in tumor tissues.

In previous studies, we showed that, among the various breast cell differentiation-associated biomarkers studied, increased α2 integrin and casein levels were found to be the most reliable and sensitive parameters indicating response to $1\alpha(OH)D_5$. We studied alpha2 integrin expression in paraffin sections of human breast carcinomas and nonmalignant breast tissues. Although the alpha2 antibody used in our studies is highly recommended for immunohistochemistry, we were unable to observe specific staining for integrin in any tissues studied. We tested several antigen retrieval systems (citrate buffer, protease digestion, trypsin digestion, SDS treatment, microwave techniques) with non-reliable results. Currently, we are analyzing alpha2 integrin expression in frozen tumor/nonmalignant breast tissues.

Studies of other prognostic markers in primary breast carcinomas

All primary breast carcinomas are analyzed for the expression of various prognostic markers (ER, PR, and p53 protein).

***In vivo* study:**

We had sufficient tumor tissue available after performing in vitro studies from 3 patients. We first developed a primary culture from the breast tumor, and then the cells growing in culture were injected into 2-3 animals. The cell suspension was mixed with Matrigel (1:1 vol.), then injected s.c. near the mammary fat pad into 3- to 4-week-old female athymic mice (at least 4 animals/tissue). Two of the 3 primary cultures developed xenografts. The xenografts were further propagated into 10 athymic mice. Animals were divided into two groups: 1) receiving control diet and 2) receiving vitamin D-supplemented (25 μg/kg-diet) diet. These experiments are in progress currently.

We have already shown that the dose selected for $1\alpha(OH)D_5$ is nontoxic and is effective for inhibiting the growth of various breast cancers transplanted into athymic mice.

Mechanism of $1\alpha(OH)D_5$ action: In vitro studies using established breast carcinoma cell lines

Previously we showed that the antiproliferative effect of $1\alpha(OH)D_5$ is more prevalent in ER+ cells and that the cell-differentiating effect is more common in VDR+ cells, irrespective of their ER status. We hypothesize that vitamin D_5 or its metabolite induces differentiation in all breast carcinomas positive for VDR, whereas in ER+ tumor, it has an additional antiproliferative effect being mediated by an interaction with estrogen receptor. We attempted, in this funding period, to answer the following questions: Is the 1α(OH)D5-mediated, antiproliferative effect in ER+ breast cancer cells due to the interaction of 1α(OH)D5 with estrogen receptors? Is the effect of 1α(OH)D5 on the induction of differentiation in both ER+ and ER- cells mediated through its interaction with VDR?

In order to achieve this aim, we used the commercially available human breast carcinoma cell line MDA-MB-231. MDA-MB-231 cells show low/undetectable levels of VDR. This cell line shows no growth inhibitory effect to $1\alpha(OH)D_5$.

During this funding period, we transfected MDA-MB-231 cells with various cDNA constructs: 1) Vector with ampicillin resistance gene only; 2) vector with human VDR cDNA construct; 3) Vector with VDRE c DNA construct; and 4) Vector with both VDR and VDRE inserts. The cDNA transfection was carried out using the Lipofectin method. Cells were subjected to G-418 resistance, and colonies resistant to G-418 were selected and expanded into cell lines. We examined the expression of VDR in all cell lines by immunohistochemistry and by western blot analysis. We are unable to observe enhanced expression of VDR in any of the transfectants.

Is the cell-differentiating effect due to its interaction with ER?

In order to achieve this goal, we have established four different cell lines, as originally proposed in the application. MDA-MB-231 cells were used in this study. In the previous report, we showed that MDA-MB-231 cells show undetectable VDR expression. In vitro, MDA-MB-231 cells fail to show growth-inhibitory response to $1\alpha(OH)D_5$. MDA-MB-231 cells transfected with full-length cDNA for human estrogen receptor were obtained from Dr. Craig Jordan of Northwestern University.

All cell lines were transfected using Lipofectin.

We have generated the following cell lines:

1. MDA-MB-231 transfected with plasmid DNA containing ampicillin-resistance gene and full-length human VDR cDNA.
2. MDA-MB-231 transfected with plasmid containing ampicillin-resistance gene only.
3. MDA-MB-231 (ER cDNA-transfected S-30) cells transfected with plasmid containing zymocin-resistance gene and full-length human VDR cDNA.
4. MDA-MB-231 (S-30) cells transfected with plasmid containing zymocin resistance gene only.

We have confirmed that VDR cDNA-transfected cell lines express VDR.

1α(OH)D_5 inhibits ER expression in S-30 (ER+) VDR-transfected cells.

In order to determine the effect of 1α(OH)D_5 on ER status, we examined ER expression immunohistochemically in S-30 cells transfected with VDR. 1α(OH)D_5 treatment inhibited expression of ER in VDR-transfected S-30 cells. These results indicated that 1α(OH)D_5 or its metabolite(s) have estrogen receptor-mediated antiestrogenic effect in breast cancer cells.

All cell lines are currently growing in culture, and we are evaluating the effect of 1α(OH)D_5 on the growth and differentiation of these cells.

Effect of 1α(OH)D_5 on expression of various genes in BT-474 cells: 1α(OH)D_5 down-regulates estrogen inducible genes.

In order to determine whether 1α(OH)D_5 or its metabolites interact with estrogen receptor and probably act as an antiestrogen, we analyzed changes in various genes in control vehicle-treated and 1α(OH)D_5-treated BT-474 cells. BT-474 cells are estrogen receptor-positive and vitamin D receptor-positive. 1α(OH)D_5 inhibits both in vivo and in vitro growth of BT-474 cells. Cells were incubated with 1α(OH)D_5 or vehicle only for four days; RNA was extracted and then subjected to microarray analysis. Table 1 lists the genes which are down-regulated significantly ($p < 0.01$) in 1α(OH)D_5-treated cells as compared to vehicle treated cells. Many of these genes are regulated by estrogen or progesterone.

Table 1

Gene name	Ratio between treated and control	Comment	References
PS2	5.7	Estrogen-inducible gene	21-30
Progesterone receptor	3.2	Estrogen-inducible gene	31-32
IGFBP-5	3.2	Estrogen-regulated	33-36
IGFBP-4	2.6	-	
Integrin alpha6	1.5	Progesterone receptor-regulated	37
Laminin receptor	1.9		-
Annexin 1	1.7	Glucocorticoid receptor-regulated protein	38

Table 2 shows a list of genes that are up-regulated in $1\alpha(OH)D_5$-treated cells.

Table 2

Name of gene	Ratio of gene expression	Comment	References
Caspase 3	1.7	Enzyme associated with apoptosis, vitamin D action	39
Alpha integrin-binding protein	1.8		-
Calcineurin-binding protein	1.8	Vitamin D-related protein	-
Nucleoporin	1.9		-
Mitochondrial thymidine kinase	1.9		-
Phospholipase C	2.0		-
Cadherin 18	3.5	Differentiation-associated protein	
PKC theta	4.6		40
Vitamin D hydroxylase	6.3	Vitamin-metabolizing enzyme	41

We further confirmed the antiestrogenic property of $1\alpha(OH)D_5$ by examining progesterone receptor protein in BT-474 cells. Our results clearly suggest that $1\alpha(OH)D_5$ inhibits the expression of progesterone receptor in BT-474 cells (data not shown).

Does $1\alpha(OH)D_5$ mediate its action through interaction with VDR?

We have examined competitive binding of $1\alpha(OH)D_5$ with $1,25(OH)_2D_3$ to pure human VDR. For determining binding of $1\alpha(OH)D_5$ to VDR, VDR ligand binding domain (VDR LBD, 20 ng/tube) was incubated with 3H-1,25(OH)2D3 (S.A. 20 mCi/mmol), rat liver nuclear extract (10 mg/tube) in the presence or absence of increasing concentrations of $1\alpha(OH)D_5$ or 1,25(OH)D3 (non-radioactive) at 4°C for 15 hrs. Following incubation, free radioactivity was removed using dextran-coated charcoal. The samples were mixed with charcoal suspension and incubated at 4°C for 20 min. The samples were centrifuged at 1200 x g for 15 min. Supernatant was mixed with scintillation fluid and radioactivity was determined using a scintillation counter. Percent of binding in the presence of unlabelled ligand was calculated as binding in the presence of unlabeled ligand divided by total binding in the absence of unlabelled ligand x 100. Our results show that $1\alpha(OH)D_5$ has 1000-fold less binding affinity for VDR than $1,25(OH)_2D_3$. These results further suggest that a metabolite of $1\alpha(OH)D_5$ is possibly responsible for the growth-inhibitory and cell-differentiating action.

At present, we are studying the metabolism of $1\alpha(OH)D_5$. Dr. Reddy from Brown University is looking into epimerization of $1\alpha(OH)D_5$ as an active metabolite of $1\alpha(OH)D_5$.

Is $1\alpha(OH)D_5$ action mediated through ER?

We examined the effect of $1\alpha(OH)D_5$ in parental MDA-MB-231 cells and in those transfected with cDNA for ER (ER alpha). The cell line showing nuclear expression of ER (S-30) was kindly provided to us by Dr. Craig Jordan, Northwestern Medical School, after obtaining written permission from Dr. J. Chambon. These series of in vitro studies were performed to

determine whether the effect of $1\alpha(OH)D_5$ is at least partially mediated through estrogen receptors.

Effect of $1\alpha(OH)D_5$ on in vitro growth of MDA-MB-231 and S-30 (MDA-MB-231 with ER) cells

For in vitro growth, cells (20,000) were plated in 24-well culture plates and then exposed to control medium (with stripped serum only) or medium containing various molar concentration of $1\alpha(OH)D_5$ or $1,25(OH)2D_3$. We also included estradiol, tamoxifen, and a combination of all these additives (shown in +s 2 and 3) as treatment groups. Medium was changed on day 4 and 7 after initiating treatment, and the number of cells was counted on day 10 using a coulter counter. Data represent mean cell number of quadruplet observations. The growth of MDA-MB-231 was not affected significantly by $1\alpha(OH)D_5$ or $1,25(OH)2D_2$ treatments. We also failed to show a significant effect of estradiol, tamoxifen, or a combination of the latter components with $1\alpha(OH)D_5$. In S-30 cells, tamoxifen was effective in inhibiting the estradiol-induced cell growth. 1,25 D3 or $1\alpha(OH)D_5$ alone had no effect on the cell growth. Interestingly, combination treatment with estradiol, tamoxifen, and $1\alpha(OH)D_5$ significantly reduced cell growth compared to that of control or estradiol alone or estradiol+tamoxifen treatment. Our results in this study suggest that $1\alpha(OH)D_5$ does not have ER-mediated action on S-30 cells. Alternatively, the S-30 cell line with exogenously transfected ER cDNA fails to respond to $1\alpha(OH)D_5$ as native ER-positive cells would. It is also possible that S-30 cells are unable to metabolize $1\alpha(OH)D_5$. Dr. Reddy is currently studying $1\alpha(OH)D_5$ metabolism in ER-positive and ER-negative breast carcinoma cell lines, including MDA-MB-231 and S-30.

In addition to growth-inhibitory action, we evaluated the effect of $1\alpha(OH)D_5$ on expression of alpha2 integrin in S-30 and MDA-MB-231 cells. Alpha2 integrin was measured by flow cytometry. In general, S-30 cells had significantly higher alpha2 integrin levels than their parental MDA-MB-231 cells . The promoter region of alpha2 integrin is reported to contain an ER-binding site (18); thus, the presence of ER even in the absence of its ligand could enhance the expression of alpha2 integrin. $1\alpha(OH)D_5$ treatment increased alpha2 integrin levels moreso than in control untreated cells. No such effect was observed in MDA-MB-231 cells.

Preclinical Toxicity under GLP:

The preclinical toxicity using two species under Good Laboratory Practice (GLP) conditions was proposed to be carried out by Dr. David McCormick as a subcontract to the IIT Research Institute, Chicago. Experiments were designed to conduct preclinical toxicity of 1α-Hydroxyvitamin D_5 using rats and dogs. Only the summary with salient features is presented here. Detailed reports were attached in the appendices in progress report for year 1 and 2.

FOUR-WEEK ORAL (GAVAGE) TOXICITY STUDY OF 1-HYDROXYVITAMIN D_5 IN RATS

Male and female CD rats (ten per gender per group) were dosed once a day for at least 28 consecutive days in order to evaluate the toxicity of the test article, 1-Hydroxyvitamin D_5 ($1D_5$). $1D_5$ was administered as a solution in corn oil by oral gavage at doses of 2.5, 5.0, and 10.0 μg/kg of body weight. A vehicle control group was administered an equivalent volume (5 ml/kg) of corn oil (vehicle). Ten rats/gender/group were necropsied after 28 (males) or 29 (females) days of dosing (Day 29 or 30, respectively). Ten additional animals in each of the vehicle control and

high-dose (10.0 µg/kg) groups were allowed a 14-day recovery period following dosing to determine the reversibility of any observed toxic effects, and were necropsied on Day 43. All animals were observed for adverse clinical signs, and body weight, body weight gain, and food consumption were measured weekly. Clinical pathology (hematology, including coagulation and red cell morphology, and clinical chemistry) parameters were evaluated at the end of the 28-day treatment period and again after 2 weeks of recovery. All rats were subjected to a gross necropsy, and tissues from control and high-dose animals sacrificed after the 28-day treatment period were processed and examined microscopically. Target tissues examined in rats from the low- and mid-dose groups and in recovery rats consisted of the kidneys and sternum.

No animals died during the study, and no adverse treatment-related clinical signs of toxicity were observed. No treatment-related effects on body weight, weekly or total body weight gain, or food consumption were observed during the study. Treatment-related, statistically significant increases in serum calcium (11.0 ± 0.46 mg/dL control vs. 11.6 ± 0.73 mg/dL high dose 1D_5) in high-dose males and (11.2 ± 0.29 mg/dL control vs. 11.9 ± 0.35 mg/dL high 1D_5) in high-dose females and in mid-dose females and in inorganic phosphorus levels (both genders of the high-dose group) were observed. Calcium and phosphorus were not increased in the recovery animals. Other statistically significant increases or decreases were noted for a few hematological and serum chemistry parameters, but were not considered treatment-related. Several other differences in absolute or relative organ weights were also observed, but were not considered treatment-related. No treatment-related gross lesions were observed at necropsy.

The increases in serum calcium seen in the high-dose males and females, and in the mid-dose females, correlated microscopically with statistically significant (p 0.05) increased incidences of mineralization in the kidney (medulla, pelvic epithelium, and/or cortico-medullary junction) of these animals. Mineralization of the renal pelvic epithelium was also present in the mid-dose males, while mineralization also occurred in the kidney of low-dose females. Other treatment-related microscopic changes seen in the kidney of females at all dose levels consisted of the presence of basophilic tubules and tubular dilatation. Degeneration of cartilage was also seen microscopically in the sternum of both genders at all dose levels. All microscopic findings were of minimal to mild severity. At the end of the recovery period, the incidence of renal pelvic epithelial mineralization was still slightly increased in both sexes, while the incidence of basophilic renal tubules and tubule dilation was still increased in the females. The change in the sternum was no longer present in the recovery rats.

In conclusion, administration of 1-hydroxyvitamin D_5 to male and female rats via oral gavage for four weeks at doses of 2.5, 5.0, and 10.0 µg/kg resulted in increased serum calcium levels in both sexes at the high-dose level and in females at the mid-dose level. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at the mid and high dose levels and in females at all dose levels. Although a no-effect level was not established in this study, the toxicological significance of microscopic lesions occurring at all dose levels was considered to be minimal because of the minimal severity of the lesions and because these lesions also occur as incidental findings in rodent studies. For further details, see toxicity report in appendix.

4 weeks preclinical toxicity studies in beagle dogs

A 28-day oral toxicity study was conducted in male and female beagle dogs to evaluate the toxicity of 1α(OH)D_5 administered by gavage for four weeks. 1α(OH)D_5 was dissolved in ethanol and then further diluted in corn oil. Four different doses were given (5, 10, 30, and 90 μg/kg body weight). Control group received vehicle at equal volume. The study design originally included 6 animals (3 male, 3 female) in lower doses (10, 30 μg) and 10 animals (5 of each sex) in higher doses.

As we observed mortality (2 dogs) in the high-dose (90 μg) group within a week of initiating treatment, the treatment dose in the remaining animals was reduced to 45 μg/kg body weight for the next 3 weeks.

Toxicological endpoints included physical examinations/clinical observations, ophthalmologic examination, body weights, food consumption, clinical pathology (hematology, clinical chemistry, urine analysis), organ weights, and electrocardiographic evaluations. Tissues from all dogs in the vehicle-treated, 10 μg dose, and 30 μg dose groups which were sacrificed were evaluated histopathologically. In addition, target tissues and gross lesions from dogs treated with the 5 μg dose were also evaluated histopathologically.

Administration of 1α(OH)D_5 at dose levels greater than 5 μg/kg induced symptoms of hypervitaminosis. Eight dogs died or were sacrificed during the study (2 females at 90 μg/kg dose, 3 males at 45 μg/kg dose, and 2 males and a female at 30 μg/kg dose).

Mean body weight and body weight gains were statistically decreased in dogs treated with >5 μg/kg dose by day 8. Body weight loss was 25-43% of their initial body weight. Body weight losses were accompanied by decreased food consumption. Erythrocyte count, hematocrit, and hemoglobin levels increased in both sexes at doses of 10 μg/kg body weight and above, which most likely resulted due to the dehydrated condition of the animals. Serum calcium levels were increased and serum inorganic phosphorus levels were significantly decreased in a dose-dependent manner in both sexes at all dose levels. In addition, females receiving 30 μg or higher doses had decreased alkaline phosphatase, along with increased blood urea nitrogen, cholesterol, and triglyceride levels.

At any dose, no treatment-related ophthalmologic or electrocardiographic changes were observed. At 5 and 10 μg doses of 1α(OH)D_5, we observed mineralization in the arteries of the spleen (females only) and heart (males only), bone marrow depletion, and cartilage hypoplasia in the femur.

In conclusion, administration of 1α(OH)D_5 at dose levels 5-90 μg/kg body weight via oral gavage daily for 28 days induced signs of hypervitaminosis. A "no observable effect level" (NOEL) was not established in this study (a detailed report is attached in the appendix).

Synthesis of 1α-Hydroxyvitamin D_5 under GMP

As proposed in the original application the synthesis of 1α-Hydroxyvitamin D_5 is being carried out by Drs. Robert Moriarty and Raju Penmasta at Conquest Inc. (formerly known as Steroids Ltd.). Dr. Moriarty has synthesized and supplied 1α-hydroxyvitamin D_5 for all our prior studies. As a part of this project, a subcontract to Dr. Moriarty is awarded for him to supply 1 gram of the compound for preclinical toxicity and 1 gram of the analog synthesized under Good

Manufacturing Practice (GMP). Dr. Moriarty already synthesized and supplied 1 gram of 1α(OH) D_5 for preclinical toxicity. Experiments described under preclinical toxicity used this newly synthesized compound. The synthesis of D_5-analog under GMP is completed. The compound is secured in UIC Pharmacy under close control of Ms Bressler, a registered Pharmacist.

Plan for the Clinical Trial

The protocol and informed consent form (both in English and Spanish) of the Phase 1/2 clinical trial is approved by UIC and currently under review by both the U.S. Army human research Regulatory Compliance and Quality review committee (Ms. Catherine A. Smith, Human Subjects Protection Specialist) and the UIC institutional review board (Dr. Eric Gislason, Vice-Chancellor). The FDA application (IND #56509) will be completed so that the current clinical hold pending these studies will be withdrawn. Once the FDA's approval is received, patient accrual will be started.

Key Research Accomplishments

Nonclinical studies:

Studies in human tumor/normal breast tissues:

The effects of in vitro 1α(OH)D5 were observed in normal breast tissues, fibroadenomas, and breast carcinomas obtained from women with confirmed diagnosis of the disease. Our results show that:

- Normal breast tissue retains the original alveolar and ductal structures when incubated in the culture medium used in this study. Breast epithelial cells appear to be normal and alive for 72 hours. All epithelial cells show VDR expression. Many appear to be proliferating, as evident from Ki-67 staining. 1α(OH)D_5 (1 μM) treatment shows no toxic effect on the breast epithelial cells; all alveolar and ductal structures are preserved. 1α(OH)D_5 has no effect on cell proliferation.
- Breast fibroadenomas retain normal structures in in vitro culture for 72 hours. Following incubation with 1α(OH)D_5, many alveolar structures show apoptotic or degenerative epithelial cells. The cells unaffected by 1α(OH)D_5 show high expression of VDR.
- Breast carcinomas treated with 1α(OH)D_5 show a significant number of cells undergoing pyknosis or apoptosis.

Studies using established human breast carcinoma cell lines:

- Our results on competitive binding studies with VDR indicate that 1α(OH)D_5 has relatively lower binding affinity than 1,25(OH)2D5. These results suggest that 1α(OH)D_5 may possibly mediate its cell-differentiating and antiproliferative actions through VDR and also through other pathways.
- We have established 4 different cell lines with different VDR and ER status. These cell lines are cloned and will be used to determine interaction between ER and VDR and the effect of 1α(OH)D_5 on these cells.
- Studies on MDA-MB-231 (ER+, VDR+) cells clearly indicate that 1α(OH)D_5 influences ER expression in breast cancer cells.

- We have further confirmed our previous findings that $1\alpha(OH)D_5$ inhibits proliferation and induces cell differentiation markers in breast tumors (tumors obtained from patients) in vitro.

Preclinical Toxicity Studies:

- We have completed the preclinical toxicity study in male and female rats under GLP. Males and females were given 1-10 μg/kg body weight $1\alpha(OH)D_5$ by oral gavage for 28 consecutive days. $1\alpha(OH)D_5$ showed no serious toxic effect. No animals died during the study, and no adverse treatment-related clinical signs of toxicity were observed. No treatment-related effects on body weight, weekly or total body weight gain, or food consumption were observed during the study. Increased serum calcium levels in both sexes at the high dose level and in females at the mid dose level. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at the mid and high dose levels and in females at all dose levels. Although a no-effect level was not established in this study, the toxicological significance of microscopic lesions occurring at all dose levels was considered to be minimal because of the minimal severity of the lesions and because these lesions also occur as incidental findings in rodent studies. The effect of $1\alpha(OH)D_5$ was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group.
- We have completed preclinical toxicity studies in dogs under GMP. $1\alpha(OH)D_5$ was tested (5-45/90 μg per kg body weight dose). The compound was given to animals daily by gavage for 28 days. At 5 μg/kg body weight dose, hypercalcemic activity was detected. The compound had some drug-related toxicity at 5 μg/kg body weight dose. All higher doses tested were toxic and hypercalcemic in dogs. Although we observed drug-related toxicity in our preclinical toxicity studies, doses tested were significantly higher than those proposed for the phase I clinical trial.

Phase I and Phase II clinical trials:

- We have prepared sufficient quantity of $1\alpha(OH)D_5$ under GMP for future clinical studies. According to the requirement of FDA, we have secured the compound at the UIC pharmacy under direct control of Ms. Linda Bressler, Registered Pharmacist at UIC Hospital.
- As a step towards initiating clinical trials, we have obtained UIC institutional IRB approval of our protocol and also informed consent form. Also, IRB approval is obtained at Lutheran General Hospital. We have completed an application for FDA approval including a detailed investigator's brochure. We are readying an application for FDA approval of the $1\alpha(OH)D_5$ phase I clinical trial for breast cancer. The brochure is prepared according to the guidelines provided to us by FDA.

Tasks originally proposed but not completed in the proposed time line and are currently under investigation:

1. Initiation of phase I clinical trial of $1\alpha(OH)D_5$ was originally proposed to initiate by last year; however, due to UIC IRB hold, it is delayed. As soon as the FDA approval is received, we aim to initiate a phase I trial. The vitamin D analog is synthesized under GMP regulations and is available for the clinical use. No cost extension letter is submitted to the DOD officials for extension of this project.

2. We have identified genes that are regulated by $1\alpha(OH)D_5$. Many of these genes are those regulated by estrogens. We are currently evaluating how $1\alpha(OH)D_5$ modulates expression of various genes.
3. Radioactive $1\alpha(OH)D_5$ is synthesized by a commercial company. We will perform pharmacokinetics study of the compound as soon as the radioactive compound is available.
4. We have crystallized the pure human VDR. Experiments are in process to determine whether vitamin D analog induces confirmational changes in VDR. These experiments will shed some light on the receptor-mediated effect of $1\alpha(OH)D_5$ in breast cancer cells.

Reportable outcomes

Publications:

1. Lazzaro G., Agadir A., Qing W., Poria M., **Mehta R.R.**, Moriarty R.M., Zhang X., Mehta R.G. Induction of differentiation by $1\alpha(OH)D_5$ in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer, 36, 780-786, 2000
2. Mehta R.R., Mehta R.G. Differentiation of human breast carcinoma cell line by a novel vitamin D analog: $1\alpha(OH)D_5$. Int J Oncology 16: 65-73, 2000.
3. Lazzaro G., Agadir A., Qing W., Poria M., Mehta R R. Moriarty R.M., Zhang X, Mehta R.G. Induction of differentiation by $1\alpha(OH)D_5$ in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur J Cancer 36: 780-786, 2000.
4. Mehta R.G. and Mehta R.R. Vitamin D and cancer. Int J Nutr Biochem, 2001, In press.

Presentations at national and international meetings:

5. Mehta R.R., Mehta R.G., Hussain E., Moriarty R., Mehta R.R. and Das Gupta T.K. Chemoprevention of mammary carcinogenesis by synthetic analog of vitamin D. Mutation Res. Seoul, Korea, 2002.
6. Johnson W.D., Mehta R.R., Moriarty R.M., Mehta R.G. Preclinical toxicity of $1\alpha(OH)D_5$ in rats and dogs. Proc Am Assoc Cancer Res 42:933, 2001.
7. Mehta R.R., Christov K and Mehta R.G. Effects of $1\alpha(OH)D_5$ are selective to malignant breast epithelial cells. Proc Am Asso Cancer Res 42:203, 2001.
8. Hussain E.A., Bhat K., Mehta R.R. and Mehta R.G. $1\alpha(OH)D_5$ induces apoptosis and cell cycle arrest in BT-474 breast cancer cells. Proc Am Assoc Cancer Res 42: 209, 2001.

Conclusions

We have completed the tasks originally proposed in the application. We have performed studies in cell lines and have completed detailed preclinical toxicity studies in dogs and rats under GLP. We have completed synthesis of $1\alpha(OH)D_5$ under GMP for future clinical trial. In vitro studies in clinical specimens obtained from women suggest that $1\alpha(OH)D_5$ has no effect on normal breast tissues; it inhibits cell proliferation in tumor cells. This implies that it has no bad effects on normal breast tissues but does inhibit cancer growth. $1\alpha(OH)D_5$ or its active metabolite possibly interacts with estrogen receptor. We will be submitting our IND application to the FDA. An investigator's brochure is prepared (see attached).

Our findings to date imply that $1\alpha(OH)D_5$ has no bad effects on an overall biologic system (beagle dog and rats) or on normal breast tissues but does inhibit cancer cell growth. The fact that we are applying for approval to bring a vitamin derivative to clinical trial represents a very hopeful development in cancer treatment.

References

1) Gudas LJ, Sporn MB, Roberts AB. Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS (eds.), The Retinoids, 2nd ed. New York, Raven Press, 1994, pp 443-520.

2) Deluca HF and Ostrem V. The relationship between the vitamin D system and cancer. Adv Exp Biol Med 206:413-29, 1986.

3) Yamazaki T, Yoshiki S, and Suda T. Differentiation of mouse myseloid leukemia cells induced by vitamin D3. Proc Natl Acad Sciences 78:4990, 1981.

4) Koga M, Eisman JA, and Sutherland RL. Regulation of epidermal growth factor receptor levels by 1,25-dihydroxy vitamin D3, in human breast cancer cells. Cancer Res 48:2734-39, 1988.

5) Taoka T, Collins ED, Irino S, and Norman AW. 1, 25(OH)2-vitamin D3 mediated changes in m-RNA for c-myc and 1,25(OH)2 vitamin D3 receptor in Hl-60 cells and related sub clones. Molec Cell Endocrinol 95:77-82, 1995.

6) Coloston KW, McKay AG, James SY, Binderup S, and Coombes RC. EB1089: A new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. Biochem Pharmacol 44:2273-80, 1992.

7) Abe-Hoshimoto, Kikuchi T, Mastumoto T, Nishii Y, Ogata T, and Ikeda K. Antitumor effect of 22-oxa- calcitrol, a new noncalcemic analog of calcitrol in athymic mice implanted with human breast carcinoma and its synergism with tamoxifen. Cancer Res 53:2534-2537, 1993.

8) Wijnggsarden T, Pols HAP, Burrman CJ, van den Bemd, JCM, Dorssers LCJ, Birkenhager JC, and van Leeuwen JPTM. Inhibition of breast cancer cell growth by combined treatment with vitamin D3 analogs and tamoxifen. Cancer Res 54:5711-17, 1994.

9) Anzano MA, Smith JM, Uskokovic MR, Peer CW, Mullen LT, Letterio JJ, Welsh MC, Shrader MW, Logsdon DL, Driver CL. 1-alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalcifer (RO24-5531), 1 new deltanoid (vitamin D analog) for prevention of breast cancer in the rat. Cancer Res 54:1653-56, 1994.

10) Elstner E, Linker-Israeli M, Said J, Umiel T, Sven V, Shintaku P, Heber D, Binderup L, Uskokovic M, and Koemer P. 20-epi-vitamin D3 analogues: A new class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 55:2822-2830, 1995.

11) Brenner RV, Shabahang M, Schumaker LM, Nauta RJ, Uskokovic MR, Evana SR, Buras

RR. The anti-proliferative effect of vitamin D analogs on MCF-7 human breast cancer cells. Cancer Lett 9285:77-82, 1995.

12) James SY, McKay AG, Coloston KW. Vitamin D derivatives in combination with 9-Cis retinoic acid promote active cell death in breast cancer cells. J Mol Endocrinol 14:391-394, 1995.

13) Koike M, Elstner E, Campbell M.J., Asou C, Uskokovic M, Tsurouks N, and Koeffler P. 19-nor-Hexafluoride analog of vitamin D3: A novel class of potent inhibitors of proliferation of human breast cell lines. Cancer Res 57:4545-4550, 1997.

14) Lopez-Boado YS, Puente XS, Alvarez S, Tolivia J, Binderup L, Lopez-Otin C. Growth inhibition of human breast cancer cells by 1,25-dihydroxyvitamin D3 is accompanied by induction of Apolipoprotein D expression. Cancer Res 57: 4091-4097, 1997.

15) Mehta RG, Moriarty R, Mehta RR, Penmasta R, Lazzaro G, Constantinou AI, Hawthorne M. Prevention of carcinogen-induced preneoplastic mammary lesions in mice in 1 a-hydroxyvitamin D5. JNCI 89:212-8, 1997.

16) Mehta RG, Hawthorne M, Uselding L, Dragos A, Moriarty R, Christov K, Mehta RR. Prevention of N-Methyl-N-Nitrosourea-induced mammary carcinogenesis in rats by 1a-hydroxyvitamin D5. JNCI 89: 212-218, 2000.

17) Mehta RR, Bratescu L, Graves JM, and Mehta RG. Differentiation of human breast carcinoma cell line UISO-BCA-4 by a novel vitamin D analog: 1((OH)D5. Int. J. Oncology 16: 65-73, 2000.

18) Lazzaro G, Agadir A, Qing W, Poria M, Mehta RR, Das Gupta TK, Moriarty RM, Zhang X, Mehta RG. Induction of differentiation by 1a(OH)D_5 in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur J Cancer 36: 780-786, 2000.

19) Johnson WD, Mehta RR, Moriarty RM, Mehta RG. Preclinical toxicity of 1a (OH)D5 in rats and dogs. Proc Am Assoc Cancer Res 42:933, 2001.

20) Mehta RR, Christov K and Mehta RG. Effects of 1a (OH)D5 are selective to malignant breast epithelial cells. Proc Am Assoc Cancer Res 42:203, 2001.

21) Loven MA, Wood JR, Nardulli AM. Interaction of estrogen receptors alpha and beta with estrogen response elements. Mol Cell Endocrinol 181(1-2):151-63, 2001.

22) Crosier M, Scott D, Wilson RG, Griffiths CD, May FE, Westley B. High expression of the trefoil protein TFF1 in interval breast cancers. Am J Pathol 159(1):215-21, 2001.

23) Allred CD, Allred KF, Ju YH, Virant SM, Helferich WG. Soy diets containing varying amounts of genistein stimulate growth of estrogen-dependent (MCF-7) tumors in a dose-dependent manner. Cancer Res 61(13):5045-50, 2001.

24) Gouyer V, Wiede A, Buisine MP, Dekeyser S, Moreau O, Lesuffleur T, Hoffmann W, Huet G. Specific secretion of gel-forming mucins and TFF peptides in HT-29 cells of mucin-secreting phenotype. Biochem Biophys Acta. 1539(1-2):71-84, 2001.

25) Liu J, Burdette JE, Xu H, Gu C, van Breemen RB, Bhat KP, Booth N, Constantinou AI, Pezzuto JM, Fong HH, Farnsworth NR, Bolton JL. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. J Agric Food Chem 49(5):2472-9, 2001.

26) Geisler J, Detre S, Berntsen H, Ottestad L, Lindtjorn B, Dowsett M, Einstein Lonning P. Influence of neoadjuvant anastrozole (Arimidex) on intratumoral estrogen levels and proliferation markers in patients with locally advanced breast cancer. Clin Cancer Res 7(5):1230-6, 2001.

27) Ulaganathan M, Familari M, Yeomans ND, Giraud AS, Cook GA. Spatio-temporal expression of trefoil peptide following severe gastric ulceration in the rat implicates it in late-stage repair processes. J Gastroenterol Hepatol 16(5):506-12, 2001.

28) Leffers H, Naesby M, Vendelbo B, Skakkebaek NE, Jorgensen M. Oestrogenic potencies of Zeranol, oestradiol, diethylstilboestrol, Bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. Hum Reprod 16(5):1037-45, 2001.

29) Diel P, Olff S, Schmidt S, Michna H. Molecular identification of potential selective estrogen receptor modulator (serm) like properties of phytoestrogens in the human breast cancer cell line mcf-7. Plant Med 67(6):510-4, 2001.

30) Reiter R, Wellstein A, Riegel AT. An isoform of the coactivator AIB1, that increases hormone and growth factor sensitivity, is overexpressed in breast cancer. J Biol Chem 2001 Aug 13 [epub ahead of print]

31) Crosier M, Scott D, Wilson RG, Griffiths CD, May FE, Westley BR. High expression of the trefoil protein TFF1 in interval breast cancers. Am J Pathol 159(1):215-21, 2001.

32) Medina D, Peterson LE, Moraes R, Gay J. Short-term exposure to estrogen and progesterone induces partial protection against N-nitroso-N-methylurea-induced mammary tumorigenesis in Wistar--Furth rats. Cancer Let 169(1):1-6, 2001.

33) Chan TW, Pollak M, Huynh H. Inhibition of insulin-like growth factor signaling pathways in mammary gland by pure antiestrogen ICI 182,780. Clin Cancer Res 7(8):2545-54, 2001.

34) Parisot JP, Leeding KS, Hu XF, DeLuise M, Zalcberg JR, Bach LA.Induction of insulin-like growth factor binding protein expression by ICI 182,780 in a tamoxifen-resistant human breast cancer cell line.Breast Cancer Res Treat 55(3):231-42, 1999.

35) Huynh H, Yang XF, Pollak M. A role for insulin-like growth factor binding protein 5 in the antiproliferative action of the antiestrogen ICI 182780.Cell Growth Differ 7(11):1501-6, 1996.

36) Dubois V, Couissi D, Schonne E, Remacle C, Trouet A. Intracellular levels and secretion of insulin-like-growth-factor-binding proteins inMCF-7/6, MCF-7/AZ and MDA-MB-231 breast cancer cells. Differential modulation by estrogens in serum-free medium. Eur J Biochem 232(1):47-53, 1995.

37) Nishida K, Kitazawa R, Mizuno K, Maeda S, Kitazawa S. Identification of regulatory elements of human alpha 6 integrin subunit gene. Biochem Biophys Res Commun 241(2):258-63, 1997.

38) Serres M, Comera C, Schmitt D. Annexin 1 regulation in human epidermal cells. Cell Mol Biol (Noisy-le-grand) 40(5):701-6, 1994.

39) Croxtall JD, Flower RJ. Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. Proc Natl Acad Sci U S A. 89(8):3571-5, 1992.

40) Park WH, Seol JG, Kim ES, Hyun JM, Jung CW, Lee CC, Binderup L, Koeffler HP, Kim BK, Lee YY. Induction of apoptosis by vitamin D3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase.Br J Haematol 109(3):576-83, 2000.

41) Brown AJ. Mechanisms for the selective actions of vitamin D analogues. Curr Pharm Des 6(7):701-16, 2000. Review.

Appendices

Appendix 1 Brochure for FDA application

Appendix 2 Publications

Publication 1

Publication 2

Publication 3

Appendix 3 Abstracts presented at various meetings

Appendix 1: Brochure for FDA application

University of Illinois at Chicago

INVESTIGATOR'S BROCHURE

1α-HYDROXYVITAMIN D5
(1α-HYDROXY-24-ETHYL-CHOLECALCIFEROL)

June 25, 2002

1α-Hydroxyvitamin D_5

Signature Page

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Table of Contents

1.0 Summary

During the past ten years, more than 400 analogs of vitamin D_3 have been synthesized with the hope of generating a non-toxic and non-calcemic vitamin D form that would be effective against cancers of various organs without having any toxic adverse effects. Only a handful of vitamin D analogs have qualified for this definition; however, none of the currently available analogs can be tolerated at high concentrations without inducing systemic toxicity. We have synthesized an analog of 24-ethylcholecalciferol (vitamin D_5), 1α-hydroxy-24, ethylcholecalciferol (1α-Hydroxyvitamin D_5 or 1α(OH)D_5), and evaluated its efficacy in experimental models.

The analog, 1α(OH)D_5, selectively inhibited growth of transformed cells without having any antiproliferative effects on normal human breast epithelial cells. It also inhibited development of carcinogen-induced precancerous mammary lesions in mice, and it inhibited cancer incidence and multiplicity of chemically induced mammary cancers in rats using two separate models. In rats and mice, it was tolerated at a much higher concentration than was the natural vitamin D metabolite was tolerated. These results suggested that this analog of vitamin D had a chemopreventive property.

1α(OH)D_5 inhibited growth of both steroid receptor-positive and -negative breast cancer cell lines. **In steroid receptor-positive cells, it induced cell arrest in G1 and apoptosis, whereas, in steroid receptor-negative cells, it induced only cell differentiation. However, for the action of 1α(OH)D_5, the presence of vitamin D receptor (VDR) is essential.** Breast cancer cells devoid of VDR do not respond to 1α(OH)D_5. The effect of 1α(OH)D_5 appears to be mediated by down-regulating estrogen-inducible genes such as progesterone receptors and pS2.

1α(OH)D_5 inhibited growth of breast cancer cells (both steroid receptor-positive and steroid receptor-negative) at less than 25 μg/kg diet concentration. This concentration can be translated as less than 250 ng per day for mice. In rat carcinogenesis experiments, the agent can be tolerated without the development of hypercalcemia at concentrations of 40 μg/kg diet (~600 ng/day, 4 μg/kg/day over a six-month period). These concentrations are much higherthan the proposed highest dose for the current Phase I trial.

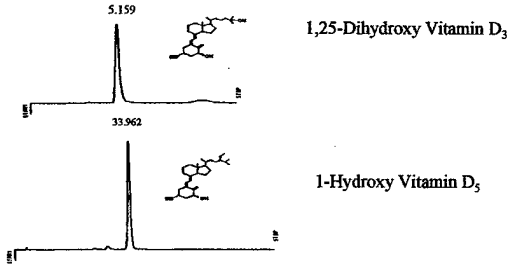
The compound for the proposed Phase I/II clinical trials was synthesized under GMP requirements and conditions by Maryfield Pharmaceuticals Inc. as a subcontract.

2.0 Introduction

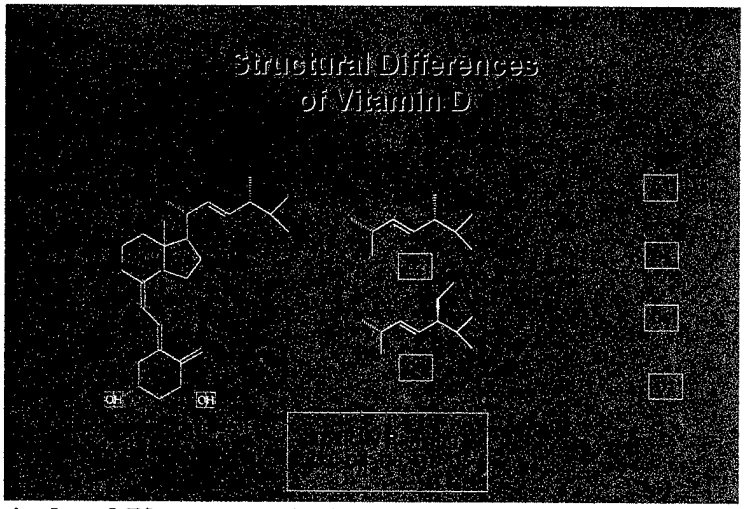
For the past 20 years, it has been well established that the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3, exhibits potent cell-differentiating properties in leukemia (1,2). The antiproliferative and differentiation-inducing effects might prove clinically significant in chemoprevention or treatment of cancer in several target organs. However, one main limitation in this type of intervention is the fact that the concentration(s) required for 1,25 dihydroxyvitamin D_3 to be efficacious is also very toxic. The effective concentration of 1,25(OH)2D3 induces dangerously high levels of serum calcium in experimental animals, resulting in body weight loss and occasionally lethality (3). This has resulted in the synthesis of analogs of vitamin D with the hope of generating an analog that is effective in prevention of cancer or suppression of growth of cancer cells without expressing any toxic effects. Typically,

the vitamin D structure is divided into four parts. The A ring, B ring, CD ring, and the side chain. Alterations can be made at any of these sites, except the modification of the CD ring is not very common due to its rigid structure. On the other hand, many alterations can be made from the open side chain. Nearly 400 analogs of vitamin D have been synthesized, and many of them have been evaluated (4,5). Only a handful of these have demonstrated potential based on in vitro and in vivo cancer models (6,7,8). The high pressure liquid chromatography (HPLC) profile of 1α(OH)D3 and 1α(OH)D5 is shown in Figure 1. The retention time for 1α(OH)D3 is 5.16 minutes, whereas for 1α(OH)D5 it was 33.96 minutes. These results indicate that these two vitamin D analogs are very different in their chemical structures. All of these analogs have been tested in a variety of cancer cell culture models,in vivo carcinogenesis models, and in xenograft models using athymic mice. The objective of such testing is to identify a compound that does not induce hypercalcemia or other undesirable effects at an effective dose level.

HPLC Profiles of Vitamin D Analogs



As shown in Figure 2, the presence of a double bond at carbons 22-23 and the presence or absence of methyl or ethyl grouping at the 24 position determine the classification of a vitamin D molecule. Thus, vitamin D is classified into six classes, which include D2, D3, D4, D_5, D6, and D7. It was reported that the vitamin D_5 class of compounds were considered as the least toxic compounds amongst all vitamin D classes (3). The double bond at 22-23, methyl group on C-24 and a hydroxy group at 3 position are characteristics of ergocalciferol (vitamin D2). Substitution of the ethyl group in place of a methyl group at C-24 makes it vitamin D6. On the other hand, D3 has no double bond at C22-C23 with a methyl group at C24 and a hydroxy group at C-3. The only difference between D3 and D5 is an ethyl group at C-24 for D5 instead of C-24 methyl for vitamin D3. Earlier, it was reported that the vitamin D_5 class of compounds were considered as the least toxic compounds amongst all vitamin D classes (3). 1α-Hydroxy-24-ethyl-cholecalciferol (1α-hydroxyvitamin D_5, 1α (OH)D_5) was synthesized and evaluated for its efficacy against experimental mammary carcinogenesis and growth of human breast cancer xenografts in athymic mice, and for toxicity. The results generated from these studies indicated that this vitamin D analog (D5) could be further developed for clinical use.

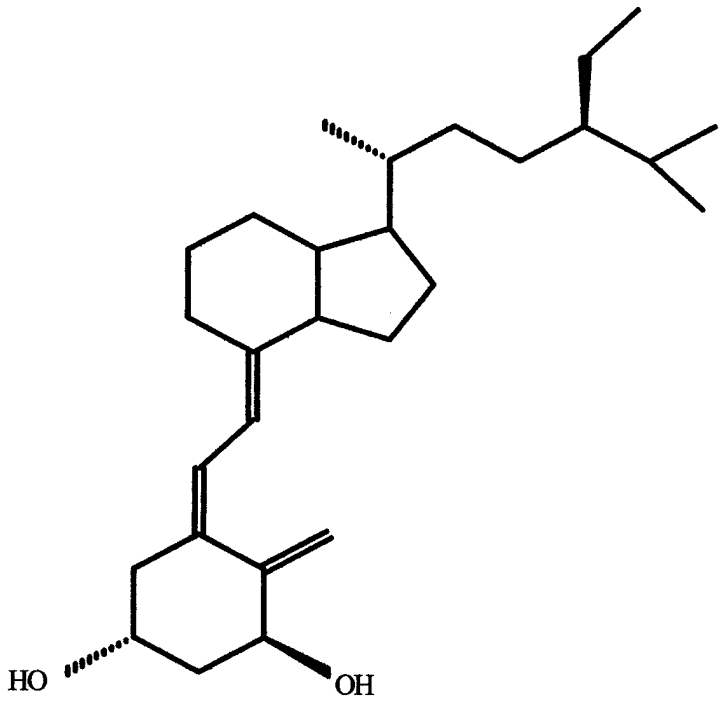


3.0 Physical, Chemical and Pharmaceutical Properties and Formulation

3.1 Chemical Information:

1α-Hydroxyvitamin D5 Structural Formula

Figure 2



Chemical Name: 1α-Hydroxyvitamin D5 is a structural analog of vitamin D5. The chemical name for it is 1α-Hydroxy-24-ethyl-cholecalciferol [1α (OH)D5].

Synthesis: The compound has been synthesized by Dr. Raju Penmasta, President, Synquest Inc., Chicago, IL, according to the Good Manufacturing Practice (GMP) guidelines.

Molecular formula: C29H48O2

Molecular Weight: 428.6

Physical form: White powder

Solubility: It is insoluble in water but highly soluble in ethanol.

Purity: The acceptable limit for the purity of the substance is 95-100%, and the analytical method used to assure the identity and purity of the compound is reversed phase HPLC. The compound 1α-hydroxyvitamin D5 was separated on C18-reversed phase 75 x 4.6 mm, 3.5 micron column, using a mobile phase of 90% acetonitrile in water. 1α-Hydroxyvitamin D5 was separated with a flow rate of 1 ml/min and monitored at 265 mμ. It was eluted with the retention time of 35 min.

3.2 Pharmaceutical Information

The compound is being formulated in the form of an oral capsule. The concentration in each capsule will be according to the protocol approved for Phase I clinical trial. This will be comparable to the oral capsule given to animals in preclinical toxicity studies under Good Laboratory Practice guidelines. The capsules for each dose level will be prepared according to the dosage schedule at the time of the initiation of the study (Table 1). This will be prepared within the Pharmaceutical Science Department in the School of Pharmacy at the University of Illinois. All the inactive ingredients in the capsule will be standard pharmaceutical components, which comply with pharmacopeal guidelines. Each capsule will contain the amount of $1\alpha(OH)D_5$ listed in the table below. **The capsules will be stored in the freezer to avoid degradation of vitamin D.** (The freezer will be kept locked, and the drug will be dispensed only by Dr. Bressler or one of her designees.) The projected number of capsules that will be required to complete the study is shown in Table 1. The plan is to prepare the capsules in batches required to complete the accrual of six patients in each of the dose groups. However, the plan may be modified as to the number of capsules prepared at any given time based on the clinical convenience and logistics.

Table 1
The projected number of capsules required for this study

Dose Level	Minimum Dose	No. Days	Total No. Capsules For 5 patients
1	5 μg/capsule/day	84	504
2	10 μg/day	84	504
3	15 μg/day	84	504
4	17.5 μg/day	84	504
5	20 μg/day	84	504
6	22.5 μg/day	84	504
7	25 μg/day	84	504

4.0 Experimental Observations

Effects of 1α (OH)D5 were evaluated in a variety of experiments. Most of these results have been published (9,10,11). Four models were employed to evaluate the efficacy of these agents. These include: 1) estrogen receptor (ER)-positive and ER-negative breast cancer cell lines; 2) mouse mammary gland organ culture (MMOC); 3) chemically induced rat mammary cancer; and 4) xenograft transplant models bearing human breast cancers. The following sections describe the results generated from these experiments.

4.1 Antiproliferative activity of 1α(OH)D_5 against well-established breast cancer cell lines in vitro.

Table 2 below shows the p53, steroid, and vitamin D receptor status of the breast cancer cell lines used in these studies.

Table 2: Steroid receptors, Her-2, and vitamin D receptor status of breast cancer cell lines

Cell line	ER status	PR status	Her-2 expression	VDR Status
MCF-7	positive	positive	low	Positive
ZR-75-1	positive	positive	medium	Positive
T-47D	positive	positive	-	Positive
BT-474	positive	positive	high	Positive
UISO-BCA-1	negative	negative	medium	Positive
MDA-MB-231	negative	negative	low	Negative
MDA-MB-468	negative	negative	Low	Negative
MDA-MB-435	negative	negative	Low	Negative
MAXF-401	negative	negative	Medium	N/A
UISO-BCA-4	negative	negative	low	Positive

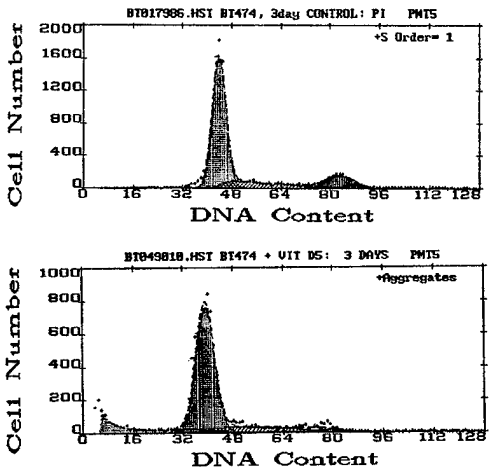
The effects of 1α(OH)D5 were evaluated on the proliferation of several breast cancer cell lines with known estrogen receptor (ER), progesterone receptor (PR), and vitamin D receptor (VDR) status. As shown in Table 2, these included MCF7 (ER+, PR+, VDR+), T47D (ER+, PR+, VDR+), ZR75-1 (ER+, PR+, VDR+), BT474 (ER+, PR+, VDR+), UISO-BCA-4 (ER-, PR-, VDR+), and MDA-MB231 (ER-, PR-, VDR-). Cells were incubated for 7 days with increasing concentrations of 1α(OH)D5 in the range of 10^{-9}M to 10^{-6}M. There was no effect observed at 10^{-9}M, and no cell toxicity was observed at the highest concentration of 10^{-6}M. On the other hand, marked toxicity was observed when cells were incubated with concentrations greater than 10^{-7}M 1,25(OH)2D3. The cells treated with 1,25(OH)2D3did not show any apoptosis but were dead and floating in the medium. When cells were stained with acrydine orange, there was no sign of

apoptosis but the cells stained orange, which is a characteristic of toxic effect of a compound. These results suggest that cells required VDR in order to be responsive to either of the two vitamin D analogs. MDA-MB-231, which is characterized by the absence of ER, PR, and VDR, did not respond at all to any of the analogs. However, the presence of steroid receptors in these cells is not essential for the antiproliferative activities observed. For example, UISO-BCA-4, which lacks both ER and PR but expresses VDR, was inhibited by 1,25(OH)2D3 and $1\alpha(OH)D_5$.

These studies demonstrated that all cell lines expressing VDR (VDR-positive) are responsive to vitamin D analog and have induced cell differentiation (9), which is characterized by increased casein and lipid expression in the cells. Furthermore, morphologically the cells exhibit signs of cell differentiation (data not shown). **Most importantly, breast cancer cells that are both ER- and PR-positive in addition to being VDR positive exhibit not only differentiation but also apoptosis.** The typical effects of 1αhydroxyvitamin D5 on the cell cycle of BT474 breast cancer cell line, which is positive for ER, PR, and VDR, is shown below in Figure 3. Flow cytometric analysis of the results showed cell cycle arrest in the G1 phase of the cycle. As compared to 70% of cells in G1 in control BT474 cells, 83% of cells were arrested in G1 in $1\alpha(OH)D_5$-treated cells.

Figure 3

Effects of D5 on the cell cycle in BT474 cells



% G-1	70.3
% S	16.1
% G-2	13.6

% G-1	83.8
% S	14.9
% G-2	1.3

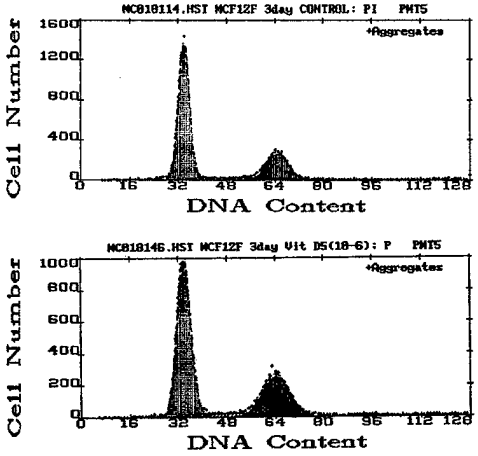
4.2 Selective effects of 1α-hydroxyvitamin D5 on transformed breast epithelial cells

A question was raised whether the effects observed against breast cancer cells would also be observed for normal breast epithelial cells. MCF12F cells are normal breast epithelial cells derived from a woman who did not have breast cancer. The cells were immortalized in culture and have been extensively used. These cells are commercially available, and this cell line was

selected for the current study. Experiments were designed to compare the effects of $1\alpha(OH)D_5$ on these normal breast epithelial cells and on MNU or DMBA-transformed MCF12F cells. The results are shown below in the Figures 4 and 5.

Figure 4

Effect of D5 on the cell cycle of MCF12F normal human mammary epithelial cells

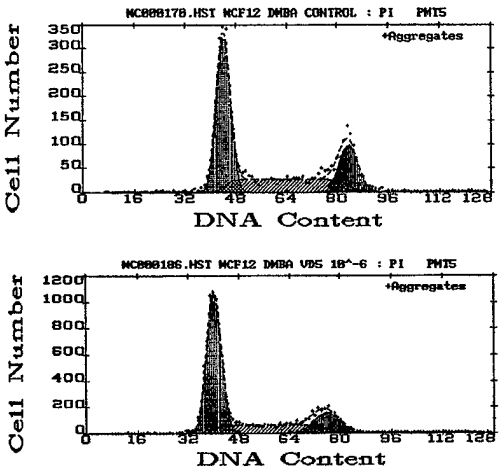


% G-1	66.8
% S	7.6
% G-2	25.6

% G-1	65.1
% S	7.8
% G-2	27.1

Figure 5

Effect of D5 on cell cycle in transformed $MCF12F_{DMBA}$ cells



% G-1	49.6
% S	29.9
% G-2	20.5

% G-1	62.1
% S	21.9
% G-2	16.0

In order to further investigate this property, the MCF12F cells were transformed with chemical carcinogens N-methyl-N-nitrosourea (MNU) and 7,12 dimethylbenz(a)anthracene (DMBA). These carcinogen-treated transformed cells have altered growth rate. $1\alpha(OH)D_5$ suppressed the proliferation of the carcinogen-treated cells, whereas the parent MCF12F cells did not respond (Figures 4 and 5). These results indicate that the effect of $1\alpha(OH)D_5$ is selective for breast epithelial cells with altered growth characteristics as observed in breast cancer cells or carcinogen-induced transformed cells.

In summary, these results demonstrate that proliferation of BT474 cells, like the other VDR+ breast cancer cells, was inhibited by $1\alpha(OH)D_5$. Moreover, cell cycle analysis showed that there was a G1 arrest in BT474 cells following exposure to 1 μM $1\alpha(OH)D_5$ for 7 days. However, there was no antiproliferative effect observed for MCF12F cells.

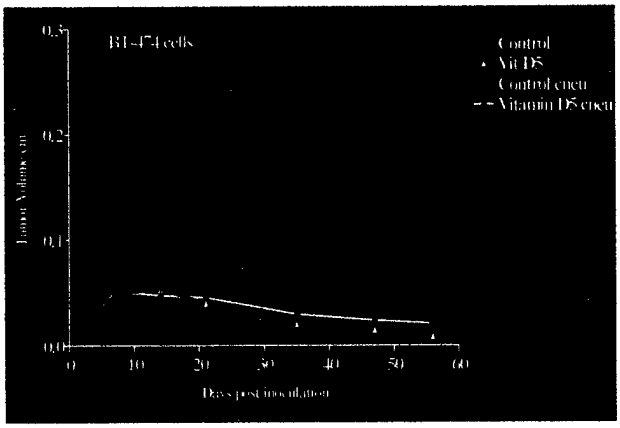
4.3 Selectivity of efficacy of 1α(OH)D5 for breast cancer tissue and not normal breast epithelium

Breast tissue samples obtained at the time of surgery of reduction mammoplasty and breast cancer samples were incubated with 1 μM $1\alpha(OH)D_5$ for 72 hours. The tissues were fixed in formalin, and tissue sections were prepared. The cell proliferative index was determined by staining tissue sections with Ki67. Results indicated that there was no effect of 1α(OH)D5 on the cell proliferation of the normal breast epithelial cells in culture, whereas the Ki67 staining was considerably reduced in the cancer tissues (data not shown). These results are consistent with the results described in previous sections for MCF12F normal breast epithelial cells and normal mammary glands in organ cultures. These histopathologic results corroborate the findings on the growth-inhibitory effects of vitamin D5 in the in vitro studies described in Section 4.1.

4.4 Effects of 1α-Hydroxyvitamin D5 on the in vivo growth of breast cancer cells in athymic mice

Previously, we reported that breast cancer cells grow more efficiently in athymic mice when they are mixed 1:1 v/v of Matrigel (10). Subcutaneous injection of 1-2 million cells into athymic mice results in the development of breast tumor. Histopathologically, these tumors are comparable to the parent cancers. We evaluated the effects of dietary modulation of $1\alpha(OH)D_5$ on the development of breast cancers of several breast cancer cell lines. These include ZR75-1, T47 D, UISO BCA-4, and MCF-7. In most of the studies, five animals per group were used. The control animals received vehicle-containing diets, whereas the powdered diet was mixed with either 10 or 20 μg/kg of $1\alpha(OH)D_5$. The mice started receiving experimental diet one day after inoculation of cancer cells. The tumor size was monitored by measuring with vernier calipers. The experiment was terminated either 60 days post inoculation with tumor cells or if the tumors reached a large size (>2 cm). Results showed that $1\alpha(OH)D_5$ suppressed the growth of breast cancer cells in athymic mice in most experiments except for MDA-MB-231 cells, which did not express VDR. An example of growth suppression of BT474 cells in athymic mice is shown in Figure 6. The results collectively suggest that $1\alpha(OH)D_5$ has a growth inhibitory role in VDR^+ human breast cancer.

Figure 6



4.5 Effects of pretreatment of breast cancer cells with 1α(OH)D_5 on subsequent development of tumors in mice

These studies were conducted with VDR-positive UISO-BCA-4 cells developed in our laboratory. The experiment was divided into two groups. In one group, the cells were treated with 1 μg/ml 1α(OH)D_5 for 10 days, and the other group served as controls. These cells were inoculated in athymic mice and allowed to grow. All five animals in the control group developed tumors, whereas there was only a scab-like lesion in the animals where the cells were pretreated with 1 μM 1α(OH)D_5 prior to inoculation. Inhibition of growth and progression of tumor in this model was attributed to 1α(OH)D_5-induced differentiation of treated cells. It can be interpreted that 1α(OH)D_5-induced differentiation in turn inhibited the growth and progression of breast cancer (9).

5.0 Mechanism of action of 1α(OH)D_5

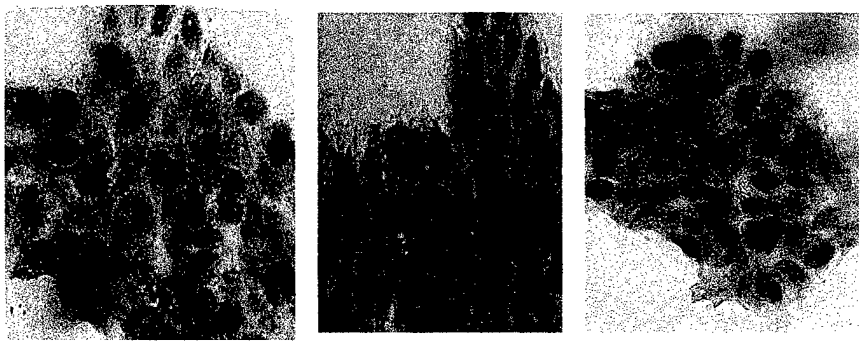
5.1 The effect of 1α(OH)D_5 is mediated by inducing cell differentiation, and VDR is essential for the function.

To examine this hypothesis, we determined the differentiating effects of 1α-hydroxyvitamin D_5 in T47D human breast cancer cells. Cells incubated with either 10 or 100 nM of the analogs inhibited cell proliferation in a dose-dependent manner, as measured by the MTT assay. This inhibition in cell proliferation was comparable to 1,25-dihydroxyvitamin D3. Both vitamin D analogs induced cell differentiation, as determined by induction of casein expression and lipid production (11). Induction of cell differentiation is often correlated with inhibition of cell proliferation. Casein and lipid expression are characteristics of normal lactating mammary glands. Thus, induction of these differentiation markers suggests that the cancer cells

are reverting to express normal function. Since the cell-differentiating effect of vitamin D is considered to be mediated via VDR, we examined the induction of VDR using RT-PCR. The results showed that, in T47D cells, both 1,25-dihydroxyvitamin D3 and 1-Hydroxyvitamin D_5 induced VDR in a dose-dependent manner. Moreover, both analogs of vitamin D up-regulated expression of vitamin D response element (VDRE)-VDR interaction as determined by CAT reporter assay (11). These results collectively indicate that 1α-Hydroxyvitamin D_5 may mediate its cell-differentiating action via VDR in a manner similar to that of 1,25-dihydroxy D3. Vitamin D analog had no effect on the expression of VDR protein in BT474 cells (Figure 7).

Figure 7

Effects of 1α(OH)D_5 on VDR Levels in BT474 Cells (Immuncytochemical Localization of VDR)



Control VD_3 10^{-7} VD_5 10^{-6}

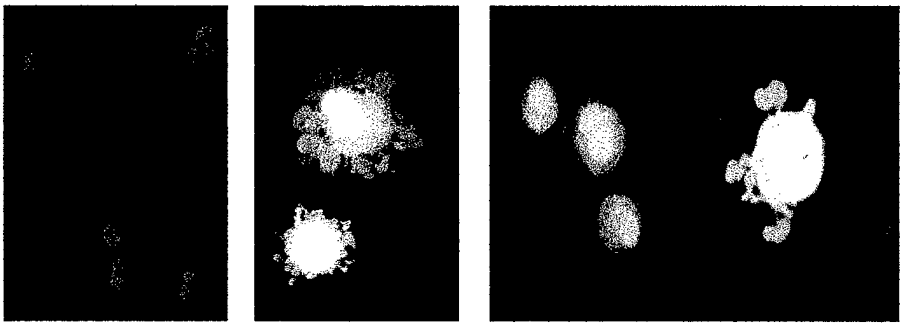
The differentiation properties of 1α(OH)D_5 were further investigated in breast cancer cells. Following 10 days treatment with 1α(OH)D_5 (10^{-7} M in UISO-BCA-4), we observed induction of intracytoplasmic casein, intracytoplasmic lipid droplets, ICAM-1, nm23, and specific biomarkers associated with breast cancer cell differentiation. 1α(OH)D_5 treatment also showed induction of vitamin D receptor and TGFβ1 proteins in the cells. These results, along with the ones described in previous sections, suggest that the action of 1α(OH)D_5 is mediated by VDR in breast cancer cells.

5.2 1α(OH)D_5 induces apoptosis in ER+, PR+, VDR+ breast cancer cells.

In ER+, PR+, and VDR+ breast cancer cells, 1α(OH)D_5 induces apoptosis as well as cell differentiation, but only cell differentiation in ER-, PR-, and VDR+ breast cancer cells. We further evaluated 1(OH)D_5-induced cell apoptosis in BT474 cells. Cell cycle analysis results indicated that, in BT474, the cell growth was arrested in G1 phase. Moreover, acridine orange/ethidium bromide staining showed apoptotic fragmentation of nuclei in these cells (Figure 8).

Figure 8

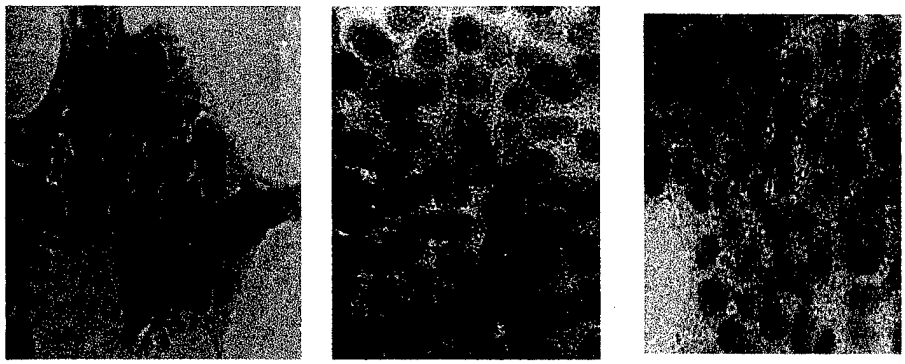
Induction of Apoptosis in BT474 Cells by $1\alpha(OH)D_5$



Control $VD_3\ 10^{-7}$ $VD_5\ 10^{-6}$

Since the only difference between these cells and BCA-4 cells was the presence of ER and PR in BT474 cells, we incubated BT474 cells with 10 nM estradiol for 5 days in steroid-stripped medium and examined estrogen-inducible expression of progesterone receptors. These cells require estradiol in the medium for cell proliferation and for the expression of estrogen-inducible genes such as progesterone receptors. As shown in Figure 9, the control cells express higher intensity of progesterone receptors. 276 cells/351 were positively stained for PgR, whereas this PgR expression was down-regulated when the cells were incubated with 10 nM estradiol plus $1\alpha(OH)D_5$ (Fig. 9).

Effects of $1\alpha(OH)D_5$ on PgR Levels in BT474 Cells



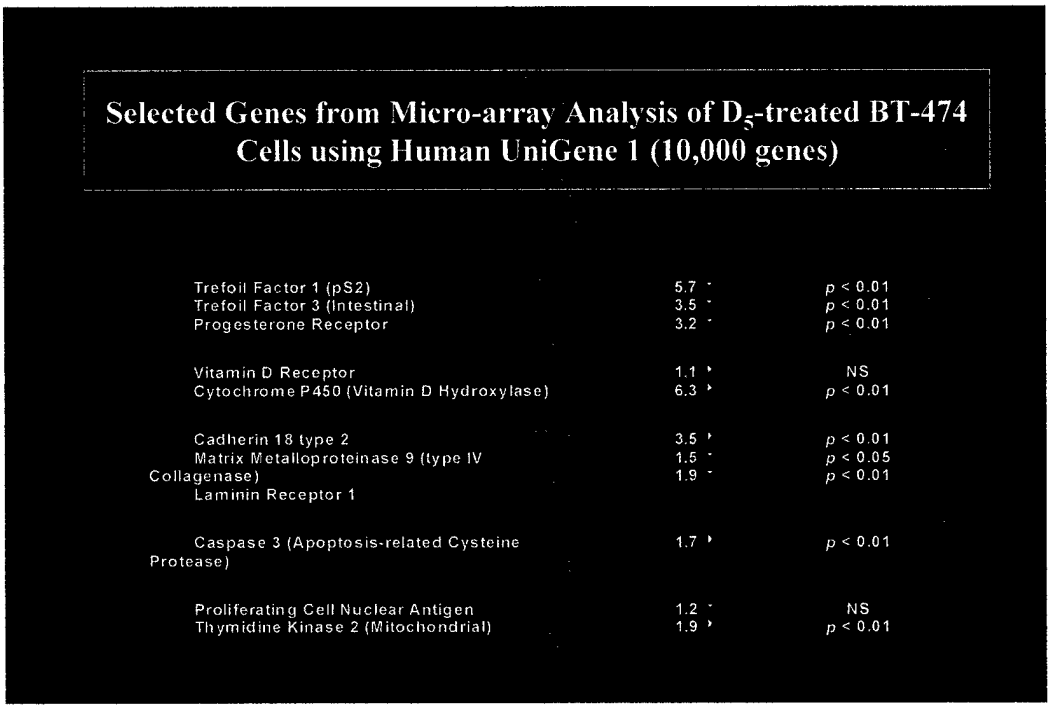
Control
276/351
++++

$VD_3\ 10^{-7}$
128/314
++

$VD_5\ 10^{-6}$
171/369
++

The effect of $1\alpha(OH)D_5$ was further determined by first determining the expression of D-altered genes by gene array analysis. Using the Unigene system, which examines a chip of 10,000 genes, mRNA prepared from $1\alpha(OH)D_5$-treated cells was compared with that of control cells. Results showed that progesterone receptors, PS2, trefoil factor, and 24-hydroxylase were some of the genes most altered by $1\alpha(OH)D_5$. These results are shown below (Figure 10). They clearly indicate that the effect of $1\alpha(OH)D_5$ in ER+ breast cancer cells is in part mediated by down-regulating estrogen-inducible genes.

Figure 10



5.3 Tissue distribution of 1α-hydroxyvitamin D_5

Tissue distribution studies have not been carried out in depth due to the unavailability of radioactive $1\alpha(OH)D_5$. We are currently in the process of having radioactive $1\alpha(OH)D_5$ synthesized. Preliminary studies were carried out to determine if $1\alpha(OH)D_5$ can be recovered from plasma, liver, and mammary tumors after 2 months of feeding with 12.5 μg/kg diet $1\alpha(OH)D_5$ in mice. The tissues were pulverized and extracted with methanol, and vitamin D metabolites were separated on a reversed-phase HPLC column. The HPLC profile showed the presence of $1\alpha(OH)D_5$ parent compound in both mammary tissues and liver. There was no peak coeluting with 1,25 dihydroxyvitamin D3. The metabolites have not been identified due to the unavailability of standards needed for identification. However, the HPLC profile showed no peak coeluting with 1,25 dihydroxyvitamin D3.

6.0 Preclinical toxicity

The main reason new analogs of vitamin D are being developed is to generate compounds with reduced or absent toxicity. The analog $1\alpha(OH)D_5$ appears to be one such relatively non-

toxic vitamin D analog. We have completed an extensive series of preclinical toxicity studies for this vitamin D analog. In this section, we describe gross toxicity, calcemic activity in vitamin D-deficient rats, and preclinical toxicity studies under GLP in two species: rats and dogs.

6.1 Gross toxicity

Treatment of animals with vitamin D analogs often results in loss of body weight gain. This is the first noticeable toxicity. As shown below, the maximum tolerated doses were determined for athymic mice, Balb/c mice, and Sprague-Dawley rats. These doses represent concentrations at which there was no loss of body weight gains and no adverse effects on general health. Lethargy, loss of body fur, loss of weight, or loss of gain of body weight are considered as signs of gross toxicity. The animals were weighed twice a week and observed daily for lethargy and other noticeable changes. However, no apparent side effects were noticed as a result of 1α(OH)D5 feeding in these animals.

Experiments were carried out to determine the maximum tolerated dietary dose of $1\alpha(OH)D_5$ for rats. Sprague Dawley rats were separated into 11 groups of 10 animals each. Group 1 served as a control. Rats in other groups received either five doses (0.8, 1.6, 3.2, 6.4, and 12.8 g/kg) of 1,25(OH)2D3 or five doses (3.2, 6.4, 12.5, 25, and 50 g/kg) of $1\alpha(OH)D_5$ for six weeks. Results showed that there was hypercalcemia and loss of body weight observed at 12.8 g/kg diet, whereas there was in fact increased body weight observed at the 50 g/kg of $1\alpha(OH)D_5$ dose level. In a separate study, no adverse effect of D5 on body weight gain was observed at 100 g/kg diet. Therefore, $1\alpha(OH)D_5$ can be tolerated at a much higher concentration than the dihydroxy-D3 analog of vitamin D.

6.2 Measurements of calcemic activity in vitamin D-deficient rats

Male rats three weeks of age were fed diet containing 0.47g% calcium, 0.3g% phosphorus, and free of vitamin D. After three weeks of consumption of this diet, serum calcium levels were measured on selected animals. Animals exhibiting serum calcium values of less than 6.0 mg/dL were considered as vitamin D-deficient. The rats were treated intragastrically with appropriate vitamin D analog for 14 days. At the end of the study, the calcium concentrations were measured in the serum. The vehicle-treated control rats showed calcium concentrations of 5.4 ± 0.3 mg/dL (mean ± standard deviation). When animals were injected with 0.042 μg/kg/day of vitamin D analogs, plasma calcium concentrations of 6.0 ± 0.6 mg /dL for $1\alpha(OH)D_5$ were observed (11% increase over control, not statistically significant from that of the control) and 8.1 ± 0.1 mg/dL for $1\alpha,25(OH)D_2$ D_3 (50% increase over control, significant increase). A higher concentration of 0.25 μg/kg/day of $1\alpha(OH)D_5$ exhibited a plasma calcium concentration of $8.1\pm$ 0.1 mg/dL as compared to $10.1\pm$ 1.8 for $1\alpha(OH)_2D_3$. Although both analogs increased serum calcium in comparison to the control samples, these results showed overall lower calcemic effects induced by **$1\alpha(OH)D_5$** as compared to **$1\alpha,25(OH)_2D_3$**. These results have been reported in publications (12,13).

6.3 Preclinical Toxicity (GLP)

Four-week oral (gavage) toxicity studies were performed on rats and dogs at the IIT Research Institute in accordance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations as set forth in the *Code of Federal Regulations (21 CFR*

Part 58). Copies of the entire document(s) for both rats and dogs experiments are attached as an appendix.

6.3.1 Rats

A 28-day toxicity study was performed in both male and female CD rats. Ten animals per sex per dose were entered in the study. 1α-Hydroxyvitamin D_5 was administered in corn oil at three dose levels: 2.5, 5.0, and 10 μg/kg of body weight. The control group of rats received only vehicle. Ten additional animals were kept in control and high-dose groups for a 14-day recovery period. All animals were observed for adverse clinical signs, body weight gain, and food consumption. Clinical pathology, hematology, and clinical chemistry measurements were carried out for every animal. Necropsy was performed in all animals, and tissues from control and high-dose animals were processed for histopathological evaluation.

No animals died from the treatment during the study. No clinical signs or adverse toxicity-related symptoms were observed at any dose level. No effect on food consumption or body weight gain were observed during the study. Treatment-related increased calcium was observed in the high-dose group (control 11.0 ± 0.46 vs. high-dose 11.6 ± 0.73 mg/dL). Calcium and phosphorus were not increased in the recovery group of animals. Increased incidence of mineralization was observed at high doses in the kidneys. All microscopic changes were of minimal to mild severity.

In summary, there was a minimal severity of mineralization observed in kidneys at the high-dose level in both sexes. These lesions often occur as incidental findings in rodent studies. Therefore, although an absolute no-effect level dose is not established, the minimal toxicity observed in these experiments might not be 1α-hydroxyvitamin D_5-related. The entire toxicity report is included in the appendix.

6.3.2 Four-week oral toxicity study in beagle dogs

A 28-day oral toxicity study was performed in both sexes of beagle dogs. 1α(OH)D_5 was administered in corn oil in 1 ml volume/kg/day at four dose levels of 5, 10, 30, and 90 μg/kg/day. The vehicle was administered in the control group of dogs. Three dogs per sex per each concentration were entered in the study. However, because of mortality in the high-dose groups, the 90 μg/kg dose level was reduced to 45 μg/kg/day for the remainder of the study. Toxicological endpoints included physical examination, clinical observations, ophthalmic examination, body weights, food consumption, hematology, clinical chemistry, electrocardiographic evaluations, and histopathological evaluations for all animals.

Eight dogs died during the study: 2 females and 3 males at the 90 μg/kg/day dose, and 2 males and 1 female at the 30 μg/kg/day dose. **Toxicity was observed at all concentrations above 10 μg/kg/ day. Serum calcium increased at concentrations at 10 μg/kg and above.** However, no ophthalmic or cardiac toxicity was observed at any dose level. In summary, the results indicated that dogs were more sensitive than rats to 1α-hydroxyvitamin D5 and that the maximum tolerated dose for this analog in dogs was 5 μg/kg/day or slightly higher but less than 10 μg/kg/day in dogs. The entire toxicity report is included in the appendix.

7.0 Synopsis of Clinical Protocol

Title: A Phase I/II Trial of 1αhydroxyvitamin D_5 ($1\alpha D_5$) in the Treatment of Metastatic Breast Cancer

Objective: To evaluate the safety and efficacy of 1αhydroxyvitamin D_5 in the treatment of patients with metastatic breast cancer

Population: Patients with metastatic breast cancer

Sample Size: 35 patients

Dosage/Treatment: Based on completed preliminary studies (described above), the first six (6) patients will receive a single daily oral dose of 1αhydroxyvitamin D_5 starting at 5 μg gelatin capsule. If there is no toxicity, the next 5 patients will be treated similarly with 10 μg daily. The dose will be escalated up to a maximum of 25 μg daily. Grade 2 (NCI Handbook) nonhematologic toxicity will define the maximum tolerated dose (MTD). Evidence of hypercalcemia will be the most important determining factor in dose escalation.

Duration: Treatment will be continued for three months (12 weeks) and/or disease/progression, though blood tests will continue for 28 weeks.

Endpoints: Safety – Clinical and laboratory adverse reactions will be closely monitored by periodic physical and laboratory examination.

Efficacy - Clinical response as measured by decrease in measurable disease determined by physical examination, radiographic studies, and/or nuclear medicine scans.

Investigators: Tapas K. Das Gupta, M.D., Ph.D., D.Sc.

Jacob Bitran, M.D.

George I. Salti, M.D.

Cathleen Schaeffer, R.N., B.S.N., O.C.N.

Details of the protocol and informed consent form are found in the appendix.

8.0 Conclusions

This report provides in-depth analysis of $1\alpha(OH)D_5$ under various experimental conditions and concludes that it is efficacious at a non-calcemic and non-toxic concentration against proliferation of cancer cells. It also shows cancer preventive activity in cell culture, organ culture, and in vivo chemically induced breast cancer models. The report also summarizes our findings relative to its possible mechanism of action and emphasizes that the action of this analog is mediated in a manner similar to that of the active metabolite of vitamin D, 1,25-Dihydroxyvitamin D3. The most important and relevant results are provided where we have

completed preclinical toxicity studies that are required as a prerequisite for Phase I/II clinical trials for breast cancer patients.

References

1. Waxman S. (2000) Differentiation therapy in acute myelogenous leukemia (non-APL). Leukemia. 14(3), 491-6.

2. James S.Y., Williams M.A., Kelsey S.M., Newland A.C., Colston K.W. (1997). The role of vitamin D derivatives and retinoids in the differentiation of human leukemia cells. Biochem Pharmacol. 54, 625-34.

3. Roder J.D., Stair E. (1999) An overview of cholecalciferol toxicosis. Vet Hum Toxicol 4, 344-348.

4. Boullion R., Okamura W.H., Norman A.W. (1995) Structure-function relationship in the vitamin D endocrine system. Endocrine Rev.16,200-257

5. Norman A.W., Song X., Zanello L., Bula C., Okamura W.H. (1999) Rapid and genomic biological responses are mediated by different shapes of the agonist steroid hormone, 1alpha,25(OH)2vitamin D3. Steroids 64, 120-8

6. Park W.H., Seol J.G., Kim E.S., Hyun J.M., Jung C.W., Lee C.C., Binderup L., Koeffler H.P., Kim B.K., Lee Y.Y. (2000) Induction of apoptosis by vitamin D3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase. Br J Haematol. 109, 576-83

7. Suzuki S., Takenoshita S., Furukawa H., Tsuchiya A. (1999) Antineoplastic activity of 1,25(OH)2D3 and its analogue 22-oxacalcitriol against human anaplastic thyroid carcinoma cell lines in vitro. Int J Mol Med 4, 611-4

8. Hansen C.M., Hamberg K.J., Binderup E., Binderup L. (2000) Secocalcitol (EB 1089): a vitamin D analogue of anti-cancer potential. Background, design, synthesis, pre-clinical and clinical evaluation. Curr Pharm Des. 6, 803-28.

9. Mehta R.R., Bratescu L., Graves J.M., Green A., Mehta R.G. (2000) Differentiation of human breast carcinoma cells by a novel vitamin D analog: 1alpha-hydroxyvitamin D5. Int. J. Oncol. 16, 65-73.

10. Mehta R.R., Graves J.M., Hart, G.D., Shilkaitis A., Das Gupta T.K. (1993) Growth and metastasis of human breast carcinomas with Matrigel in athymic mice. Breast Cancer Res. Treat., 25, 65-71.

11. Lazzaro G., Agadir A., Qing W., Poria M., Mehta R.R., Moriarty R.M., Das Gupta T.K., Zhang X.K., Mehta R.G. (2000) Induction of differentiation by 1alpha-hydroxyvitamin D(5) in T47D human breast cancer cells and its interaction with vitamin D receptors. Eur. J. Cancer 36, 780-786.

Appendix 2: Publications (3)

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Vitamin D and cancer

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Abstract

Vitamin D, a steroid hormone and exerts its biological effects through its active metabolite 1α, 25 dihydroxyvitamin D3 [1,25(OH)2D3]. Like steroid hormones, 1,25(OH)2D3 is efficacious at very low concentrations and serves as a ligand for vitamin D receptors (VDR), associating with VDR very high affinity. Despite its potent property as a differentiating agent, its use in the clinical practice is hampered by the induction of hypercalcemia at a concentration required to suppress cancer cell proliferation. Therefore nearly 400 structural analogs of vitamin D3 have been synthesized and evaluated for their efficacy and toxicity. Among these analogs, relatively less toxic but highly efficacious analogs, EB1089, RO24–5531, 1α-hydroxyvitamin D5 and a few others have been evaluated in a preclinical toxicity and in Phase I clinical trials for dose tolerance in advanced cancer patients. Clinical trials using vitamin D analogs for prevention or therapy of cancer patients are still in their infancy. Vitamin D mediates its action by two independent pathways. Genomic pathway involves nuclear VDR and induces biological effects by interactions with hormone response elements and modulation of differential gene expressions. Evidence also suggests that vitamin D analogs also interact with steroid hormone(s) inducible genes. The non-genomic pathway is characterized by rapid actions of vitamin D. It involves interactions with membrane-VDR interactions and its interactions with protein kinase C and by altering intracellular calcium channels. Thus, the development of nontoxic analogs of vitamin D analogs and understanding of their molecular mechanism(s) of action are of significant importance in the prevention and treatment of cancer by vitamin D.

Keywords: Vitamin D; Analogs of vitamin D; VDR; Carcinogenesis; Metabolism; Mechanism of action

1. Background

Vitamin D was discovered by Edward Mellanby in 1919 during his classic experiments with rickets [1]. It is a family of compounds consisting of 9,10 secosteroids, which differ, in their side-chain structures. They are classified into five forms [2]; vitamin D2, ergosterol; D3, cholecalciferol; D4, 22,23 dihydroergoalciferol; D5 sitosterol (24-ethylcholecalciferol) and D6 stigmasterol (Fig. 1). Vitamin D is derived from a cholesterol-like precursor, 7-dehydrocholesterol. When human skin is exposed to sunlight, the UV-B photons (between 290–315 nm) interact with 7-dehydrocholesterol causing photolysis and cleavage of the B-ring of the steroid structure, which upon thermoisomerization results into a secosteroid [3,4]. In order to produce physiological activity, vitamin D has to be metabolized. Numerous in-depth reviews focusing on the metabolism of vitamin D have been published. Since the metabolism of vitamin D is not the primary focus of this article, a simplistic overview of D-metabolism is briefly discussed here. The pro-hormone vitamin D gets metabolized to 25-hydroxyvitamin D in liver by 25-hydroxylase. This metabolite is present in the circulation at a concentration of more than 0.05 μM (20 ng/ml). The active metabolite of vitamin D, however is generated by hydroxylation of 25-hydroxyvitamin D at 1α-position in kidney. The enzyme 1α-hydroxylase has also been shown to be present in keratinocytes and prostate epithelial cells, suggesting that the fact that target organs may also be able to generate 1,25 dihydroxyvitamin D3 from 25-hydroxyvitamin D3 [5]. More recently mRNA for 25-hydroxvitamin D-1α-hydroxylase has been reported in normal and malignant colon tissue [6,7]. The active metabolite 1α,25-dihydroxyvitamin D is present in the human plasma at a concentration of 0.05–0.15 nM (20–60 pg/ml) [8,9]. In addition to 1α-hydroxylation of 25-hydroxyvitamin D3, many metabolites have been identified. These metabolites are side chain modifications with no definitive function assigned to them. The overall path of metabolism of vitamin D2 is similar to vitamin D3 with a few differences [10]. Both 25-hydroxyvitamin D2 and 1α,25-dihydroxyvitamin

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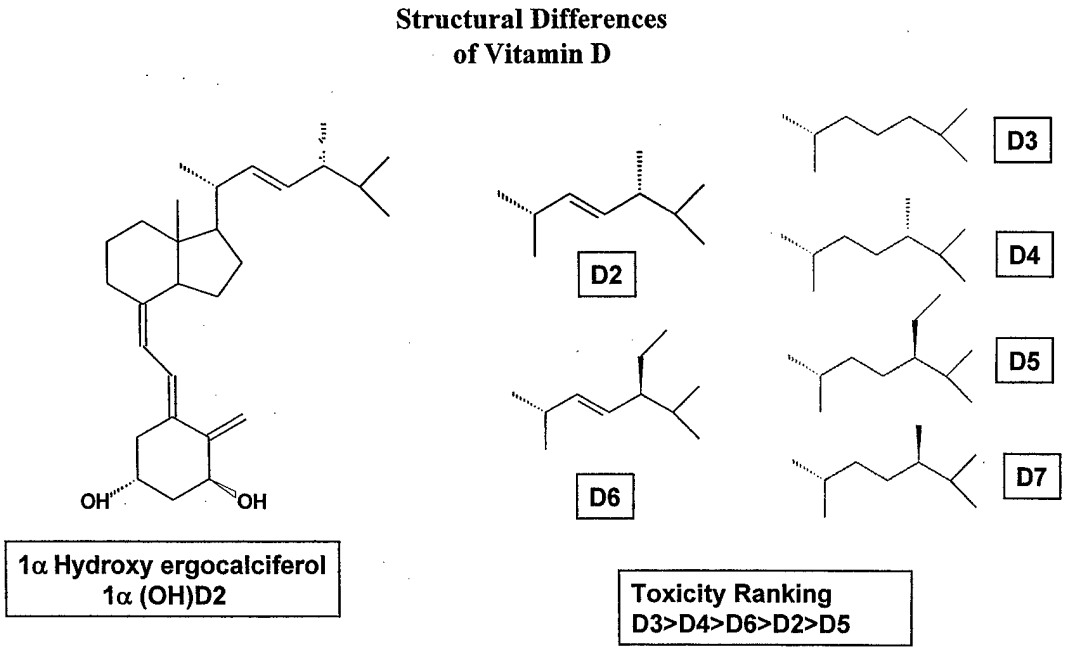

Fig. 1. Structural differences of vitamin D. Vitamin D has been classified into various classes of D2 through D7. Ergocalciferol is classified as vitamin D2 and vitamin D6 is a 24-ethyl analog of vitamin D2. On the other hand vitamin D3 or cholecalciferol is modified by either methyl or ethyl group on C-24 position. These vitamin D molecules are further classified as D3, D4, D5 and D7.

D2 have been evaluated for their biological functions. The catabolism of vitamin D occurs by further hydroxylation of 25-hydroxyvitamin D3 by 24 hydroxylase to yield 24,25 dihydroxyvitamin D3. The enzyme 24-hydroxylase is ubiquitous and is expressed in all the cells expressing vitamin D receptors (VDR). The enzyme is regulated by PTH and 1α,25-dihydroxyvitamin D3. The major significance of 24-hydroxylation is inactivation of vitamin D3 [11,12]. The inactivated vitamin D metabolites are nonfunctional. The overall metabolism of vitamin D is outlined in Fig. 2.

2. Experimental basis for vitamin D and cancer

For the past 20 years it has been consistently reported and well established that the active metabolite of vitamin D, 1,25(OH)2D3 exhibits potent cell differentiating property in leukemia cells as well as much cancer cells [13,14]. The antiproliferative and differentiation-inducing effects can be of clinical significance in prevention or treatment of cancer of several target organs. One of the main limitations in this modulation is the fact that the concentration required for being efficacious for 1,25(OH)2D3, is also very toxic. The effective concentration of 1,25(OH)2D3 induces dangerously high levels of serum calcium in experimental animals resulting in body weight loss and could be occasionally lethal [15]. This has resulted in the synthesis of analogs of vitamin D molecule with the hope of generating an analog that is effective in prevention of cancer or suppressing growth of cancer cells in culture and in vivo models without expressing any toxic adverse effects. Typically, the vitamin D structure is divided into four parts. The A ring, B ring, CD ring and the side chain. The alterations can be made at all these four sites, except the modification of the CD ring is not very common due to the rigid structure. The maximum alterations, on the other hand, are made from the open side chain. Nearly 400 analogs of vitamin D have been synthesized and many of them have been evaluated [16,17]. As far as the efficacy in in vitro or in vivo cancer models are concerned, where the risk benefit ratio related to toxicity and efficacy is determined, only a handful of vitamin D-chemicals have been successfully utilized [16,18]. The most widely studied analogs besides 1,25-dihydroxy D3, include 22-oxa-calcitriol [19,20] (Chugai Pharmaceuticals, Japan), EB1089 [21] (Leo Pharmaceuticals, Denmark), calcipotriol, KH1060 [22] (Leo Pharmaceuticals, Denmark), R024–5531 [23,24] (Hoffman la Roche, Nutley, NJ) and recently synthesized analog from our laboratory, 1α-hydroxy-24 ethyl-vitamin D3 [25] (1α(OH) D5, OncQuest, Chicago, IL). The side chain modifications of vitamin D3 molecule to result in these selective structures is shown in Fig. 3. All these analogs have been evaluated in a variety of cancer cell culture models, in vivo carcinogenesis models and in xenograft models using athymic mice. The main selection criteria here is to adopt a compound that does not induce hypercalcimia or other undesirable side effects at the effective dose level. The criteria for selection of vitamin D agents for other conditions such as bone disease, immunomodulation or hormonal therapy or nutrition can be very different and will not be discussed here since it will not be within the scope of this review.

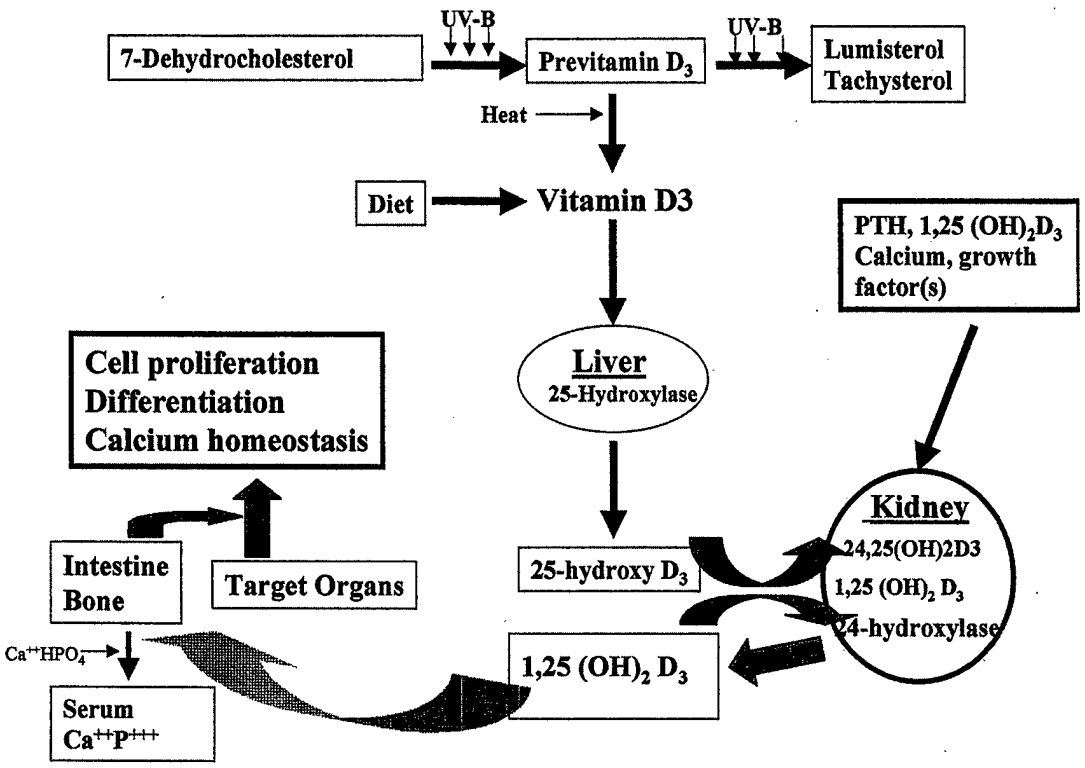

Fig. 2. Metabolism of vitamin D. Conversion of 7-hydrocholesterol to previtamin D3 by UV light and its subsequent processing to vitamin D3 and active metabolite 1,25-dihydroxyvitamin D3 is schematically shown. Vitamin D metabolism by liver and its processing by kidney is also shown in this diagram.

2.1. *Efficacy of vitamin D analogs on breast cancer in vitro*

Effects of vitamin D analogs on cell proliferation has been studied in a number of breast cancer cell lines as well as on the cells derived from many other target organs. The breast cancer cell lines expressing estrogen receptor (ER+) as well as ER-status have been utilized. All the analogs evaluated thus far have shown antiproliferative effects on ER+ breast cancer cells [26]. However, the effects of vitamin D on the ER- cells are not consistent. 1α(OH)D5 is effective against ER- BCA-4 cells whereas it is ineffective against ER-BCA1 and MDA-MB 231 and MDA-MB-468 cells [27,28,29]. The MDA-MB cell lines express vitamin D receptor poorly. Presence of low expression of VDR and absence of VDR in these cells has been reported. On the other hand, all ER+ cell lines express VDR and are responsive to vitamin D analogs (Table 1). We and others have shown that except for some ER+ breast cancer cells such as MCF-7 and BT474 cells, vitamin D analogs do not induce apoptosis [30,31]. The majority of the cells respond to vitamin D by induction of cell differentiation. Induction of cell difefrentiation is analyzed by cell morphology, flow cytometry, lipid expression and expression of casein and integrin α2 in breast cancer cells [27]. Table 1 summarizes the effects of all the commonly used analogs of vitamin D. The majority of the analogs showed efficacy against ER+ cells at noncalcemic concentrations that are greater than 1,25(OH)2D3. Among the agents effective against ER- cells, KH1060 and 22-oxa-calcitriol appeared to be very effective against MDA-MB-231. KH1060, however was not effective against MDA-MB-435. This is especially interesting since MDA-MB-231 cells are reported to have either no VDR or very low expression of VDR. Both KH1060 and 22-oxa-calcitriol have similar chemical alteration at C-22 position and both are effective against MDA-MB-231 cells. Thus may provide an altered mechanism of action that may not involve VDR or estrogen responsiveness. Other efficacious analogs including 1α(OH)D5 are not effective in VDR- breast cancer cells (Table 1).

In addition to cell culture models experiments have been carried out in mammary gland organ culture model. Mouse mammary gland responds to carcinogen in the presence of growth promoting hormones and form precancerous alveolar or ductal lesions [32]. It has been shown that transplantation of epithelial cells prepared from these glands form adenocarcinoma in syngeneic mice [33]. This model has been used for studying efficacy of chemopreventive agents and understanding mechanism of their action. Comparison of 1,25-dihydroxyvitamin D3, RO24–5531 and 1α-hydroxyvitamin D5 indicated that the D5 analog exhibited similar activity compared to dihydroxy D3 at a log molar higher concentration. RO24–5531 and EB 1089 were toxic at concentration higher than 1 μM [25], whereas 1α-hydroxyvitamin D5 can be tolerated at higher concentrations. The analog 1α(OH)D5 induced VDR and TGFβ in the

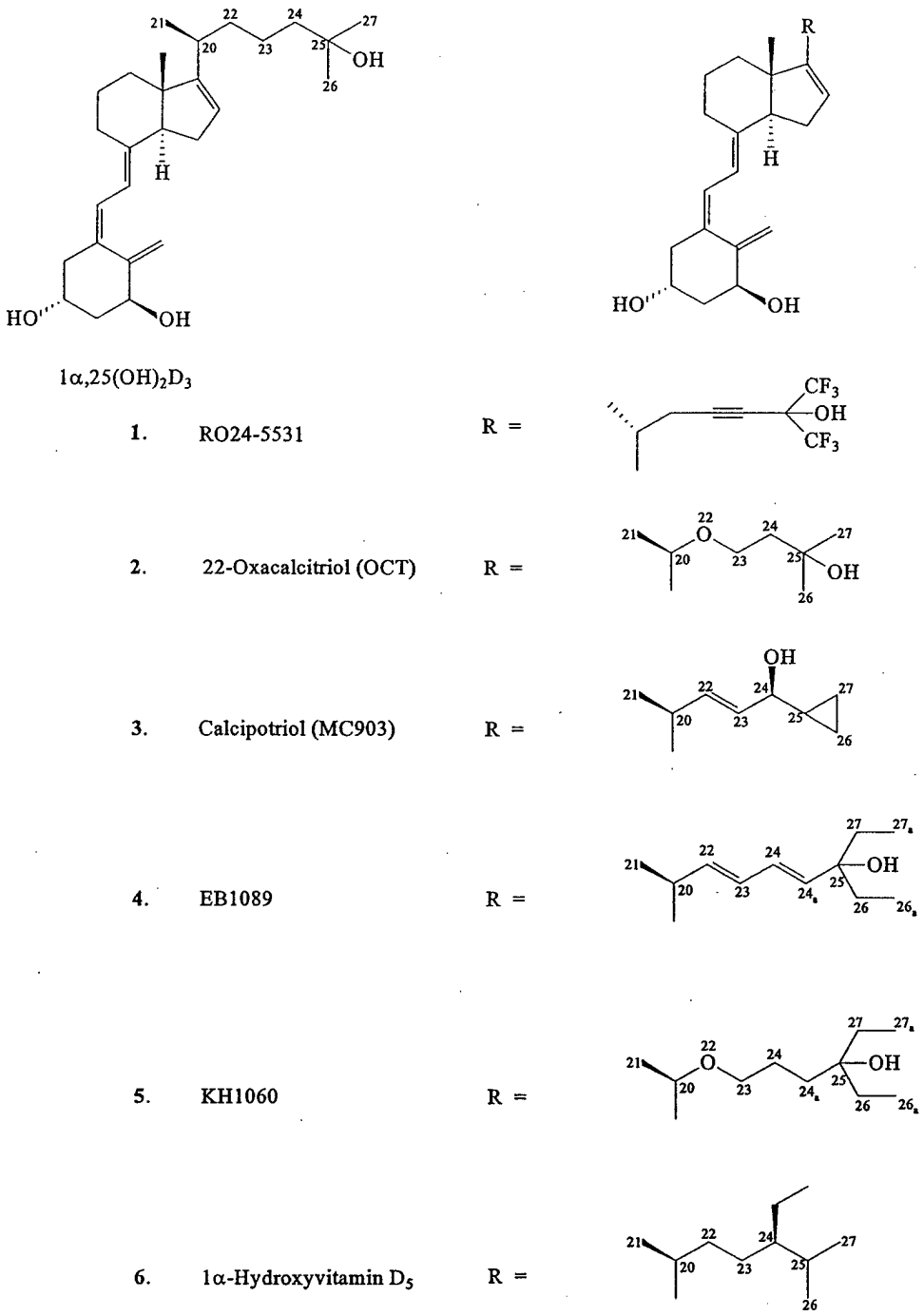

Fig. 3. Chemical structures of some of the active analogs of vitamin D.

mammary glands. These results suggested that the inhibitory effect of vitamin D analog 1α(OH)D5 be mediated by VDR.

2.2. Vitamin D and other cancers

Effects of vitamin D analogs have been evaluated in a number of cell types. The majority of cancer cell types, including HL60 leukemia, Coco and HT29 human colon cancer cells and a variety of prostate cancer cells including LnCap cells are all responsive to vitamin D analogs [34]. More recently it was noted that the incubation of prostate cancer cells as well as normal prostate epithelial cells express 1α-hydroxylase activity which is responsible for converting 25-hydroxyvitamin D3 to the active metabolite 1,25-dihydroxyvitamin D3 [35,36]. Since 25-hydroxyvitamin D3 is less calcemic and less toxic compared to the dihydroxyvitamin D3, it may be more suitable for prostate cancer prevention and therapy. Moreover, all prostate can-

Table 1
Summary of efficacy of vitamin D analogs in cancer cell prolifration

Target organ	Cells	Vitamin D analogs	Efficacy	Comments
Breast	ER+			
	MCF-7, ZR75-1, T47D BT474, BT20, SK-BR-3	22-oxa-calcitriol, 1α(OH)D_5EB-1089, KH11060, MC903, RO24-5531, 22-oxa-Calcitriol	All effective	VDR+
	ER-			
	MDA-MB-231, MDA-MB-436	1α-(OH)D_5, 22-oxa-calcitriol, KH(1060, RO24-5531	Ineffective	VDR+/−
	UISO-BCA-4	1α(OH)D_5	Effective	VDR+
	UISO-BCA-1	1α-(OH)D_5	Ineffective	VDR−
	MDA-MB-231,	22-oxa-calcitriol	Effective	VDR+/−
Prostate	LnCap, PC-3	1α(OH)D_5, EB1089, RO24-2637, 22-oxa-calcitriol, MC903	All Effective	VDR+
	Du-145	1,25$(OH)_2D_3$, RO23-7553	Ineffective	VDR+/−
	Du-145	RO24-5531, RO26-2198	Effective	
Colon	HT-29, CaCo-2	1,25$(OH)_2D_3$, RO24-5531	Effective	VDR+

cer cells expressing positive efficacy for viamin D analogs are VDR positive. It has also been reported that the low VDR expresser PC3 and DU 145 cells poorly respond to the vitamin D as compared to LnCap cells. However, transfection of VDR cDNA was sufficient to establish growth responsiveness in PC3 and DU 145 cells. These results suggest that the presence of VDR is essential for the responsiveness of vitamin D, however the content may not directly correlate with the efficacy of the analog in prostate cells [37]. At the same time, the efficacy of vitamin D analogs did not correlate with the affinity of binding to VDR [38]. The majority of the analogs express lower affinity for VDR as compared to 1,25-dihydroxy D3, and yet they inhabited cell proliferation as effectively as the active metabolite. As with other compounds, 1α(OH)D5 also inhibited LnCap cell growth at 10–7 M concentration (unpublished). These results indicate that besides VDR, other factors may also influence action of vitamin D in cancer cells (Table 1).

2.3. Efficacy of vitamin D analogs in vivo

In order to establish possible clinical significance of vitamin D in preventing or treating cancer, it is essential to evaluate its activity in experimental models. Although over the past several years there is a considerable effort diverted towards evaluating chemopreventive effects of analogs of vitamin D in carcinogen induced experimental tumor models, very little mechanistic studies have been carried out. Unlike homogenous cell type in tissue culture, in vivo studies are much more complex and there is heterogeneity of cell types and presence of tissue interactions. Nonetheless, it is extremely important to establish the role of a chemopreventive agent in carcinogenesis models prior to understanding its mechanism(s) of action. Here, we have summarized current literature regarding the protective effects of analogs of vitamin D. The prerequisites for chemoprevention experiment are to ascertain that the agent is effective at a non-toxic concentration [29]. One of the primary side effect of vitamin D is hypercalcimia because of vitamin D treatment [39,40]. Therefore, the agent has to be active at non-hypercalcimic concentration. It is also important to mention that some analogs may be non-calcemic and yet can not be tolerated at high concentrations. Therefore, in such cases it is necessary to monitor the toxicity of the agent in a dose response study. This is usually achieved by establishing a maximum tolerated dose for each chemopreventive analog of vitamin. So far, there are only a handful of analogs evaluated in vivo for their efficacy in chemoprevention. These include RO24–5531 (Hoffman-LaRoche), EB 1089, CB 966, MC903 (Leo Pharmaceuticals), 22-oxacalcitriol (Chugai Pharmaceuticals Japan) and 1α(OH)D5 (OncQuest Inc.). Although experimental models for carcinogenesis are available for several target organs, effects of vitamin D analogs have been studied mainly in mammary and colon carcinogenesis with sparse reports on a few other organs. The results are summarized in Table 2.

2.4. Mammary carcinogenesis

The most widely utilized models are 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (MNU). Both carcinogens induce mammary adenocarcinoma in rats with nearly 100% incidence. The time course of appearance of tumors and their response to ovarian hormones is well worked out [41]. Nearly all the tumors induced by MNU are ovarian hormone dependent whereas 80% of the tumors developed in response to DMBA are hormone dependent. The other 20% tumors induced by DMBA are fibroadenoma. The histopathological evaluations reveal very close similarities between tumors induced by these carcinogens in rats and human breast cancer pathology. These tumor models are extensively used for evaluation of chemopreventive agents for their efficacy [42]. In earlier studies it was ob-

Table 2
Summary of efficacy of vitamin D analogs in chemical carcinogenesis models

Organ	Models	Analog	Dose	Efficacy	Comments
Breast	MNU-induced adenocarcinoma	RO24-5531,	1,10 nmole/kg diet	Effective	No toxicity
		1α-Hydroxyvitamin D_5	58.4, 116.8 nmole/kg	Effective	No hypercalcemia
				Dose related effect	No loss of body weight
		1α-hydroxy D_3	0.25 nmole	growth inhibition	Treatment schedule
		$1,25(OH)_2D_3$	0.59-2.99 nmole/kg	No Effect	Hypercalcemia
		MC903	111 nmole/kg	Growth inhibition	Hypercalcemia
		EB1089	1.1-5.5 nmole/kg	Effective	Hypercalcemia
					Loss of body weight
Prostate	MNU-induced	RO24-5531	10 nmole/kg	Effective	No toxicity
					No effect on dorsal prostate
		1α(OH)D_5	58.4-116.8 nmol/kg diet	In progress	
Colon	AOM-induced	RO24-5531	2.5 nmole/kg ip	Effective	No toxicity
	DMH-induced	22-oxa-Calcitriol	72.5 nmole/kg ip	Effective	
	DMH-induced	24R,25 dihydroxyvitamin D_3	0-24 nmole/kg	Effective	Reduced aberrant crypt
	DMH, MNU, and nitrosamines	24R,25 dihydroxyvitamin D_3	0-12 nmole/kg	Effective	foci colon only

served that treatment with 1,25 dihydroxyvitamin D3 up to 3 nmole/kg BW of rat resulted in no protection against mammary carcinogenesis and yet increased calcium levels in blood was reported [43,44]. Two other vitamin D analogs studied in this report included EB1089 and MC 903. MC903 at a very high dose provided some prtection against mammary tumor growth whereas EB1089 was effective at all the doses evaluated [45,46]. However, there was hypercalcimia observed at <2 nmole (1 and 2.5 μg)/kg dose level. An in depth study to evaluate effects of R024–5531 against MNU-induced mammary carcinogenesis has also been reported. Anzano et al showed that this non-calcemic analog was effective against both the incidence and multiplicity of mammary tumor development at very low levels of 2.5 nmole per kg of diet [24]. However, this effect was observed only when low carcinogen dose was employed. At higher carcinogen dose, level there was no effect against the tumor incidence. Higher than 2.5 nmole per kg of diet dose level was not evaluated in this study, it is possible that it induces toxicity other than hypercalcimia at higher concentrations and 2.5 nmole may in fact be maximum tolerated dose in rats. In a more recent study, we evaluated effects of 1α-hydroxyvitamin D5 in MNU-induced mammary tumor model. The results showed that the animals could tolerate 116 nmole/kg (50 μg/kg) diet concentration of the analog during a six-week toxicity study without adversely affecting serum calcium levels. In older animals, dietary treatment with 0.116 μmole/kg diet 1α-hydroxyvitamin D5 reduced both the incidence and multiplicity of MNU-induced mammary tumors [47]. In this experiment, the vitamin D supplementation began two weeks prior to the carcinogen treatment and continued through out the experiment (Table 2). This meant that both initiation and promotion phases were not separated and the dietary modulation was included during both phases. The selectivity between these two stages in relation to 1α-hydroxyvitamin D5 effect is currently in progress.

2.5. Colon carcinogenesis

There are several well-established colon carcinogenesis models available for evaluating effects of chemopreventive agents. Carcinogens successfully utilized for induction of colon cancers are MNU, 1,2, dimethylhydrazine and azoxymethane. The time frame of induction of aberrant crypts and carcinomas of colon by DMH and AOM have been established [48]. Analogs 1α-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 have been used against DMH induced colon carcinogenesis. Rats received 20 weekly injections of 20 mg/kg DMH. 1,25, Dihydroxyvitamin D3 at a concentration of <0.3 nmole reduced the incidence of colon carcinomas from 46% to 11%. However, there was hypercalcimia associated with this efficacy. In a separate study, effect of R024–5531 was also studied in colon carcinogenesis. Dietary inclusion of R024–5531 for 34 weeks resulted in 40% reduction of colon cancer formation in treatment groups [49]. None of the tumors developed in vitamin D treated rats was adenocarcinoma, they were all benign. Thus, RO24–5531 appears to be very effective against colon cancers. In another study, Otoshi showed that the IP injections of 22-oxa-cxalcitriol also suppressed the development of aberrant crypt foci in rats [50]. The analog, 1α-hydroxyvitamin D5 has not been evaluated for its efficacy in colon carcinogenesis (Table 2).

2.6. Transplantable models

Unlike chemically induced carcinogenesis models, transplantable models are used to evaluate effects of test agents on the growth of the established tumor cells. Since these studies are largely conducted with human cancer cells growing in culture, athymic mice are used as animal of choice. Surprisingly, not all cancer cells form tumors in athymic mice Earlier we had reported that breast cancer cells mixed with matrigel in the ratio of 1:1 results in a remarkable

increase in the development of tumors. Since our original report [51], the use of matrigel for better response in athymic mice has become a common practice for breast cancer. On the other hand, melanoma, sarcoma, colon cancers and prostate cancers typically are not mixed with matrigel to grow in nude mice. Effects of 1,25 dihydroxyvitamin D3 and synthetic analogs of vitamin D3 have been evaluated for their anticancer efficacy on the growth of many cancer types. Studies from our laboratory have shown that 1α-hydroxyvitamin D5 inhibited growth of steroid receptor positive MCF-7 as well as ZR75A cells in vivo [27]. Both these cell lines are positive for both ER and VDR. Cell line established in our laboratory, BCA-4, which is positive for VDR but negative for estrogen and progesterone receptors also responded to the D5 analog of vitamin D. The responsiveness was observed at 0.3nmole i.p. injections or by dietary incorporation of 30 nmole D5-analog/kg diet. These results suggested that the presence of VDR was essential for the efficacy of vitamin D analogs and steroid receptors were of less significance. This was further confirmed by demonstrating lack of effect of 1α (OH) D5 in MDA-MB231 cells that either lack VDR or are relatively very low in expression of VDR expression [27,52].

Effects of 1,25-dihydroxyvitamin D3 was evaluated and compared with EB1089 in transplantable prostate tumor model using androgen-insensitive metastatic rat prostate model. MAT LyLu cells were injected in Copenhagen rats and appropriate groups were treated with low (0.5 μg/kg) and high (1 μg/kg) doses. Both these analogs reduced the metastatic foci in lungs in these rats, however the effect was accompanied by hypercalcemia and loss of body weight at higher dose [53]. More recently, we evaluated effects of 1α(OH) D5 on the growth of LnCap cells in athymic mice (unpublished). Results showed that 55 nmole/kg (25 μg/kg) of diet of the vitamin D analog for 60 days resulted in reduced tumor volume as compared to the control LnCap tumors. At 55 nmole/kg diet concentration, the D5 analog did not elevate concentration of serum calcium levels. Thus, the experimental evidence indicates that not only vitamin D analogs are effective as chemopreventive agents in experimental carcinogenesis models but they also suppress the growth of human cancer cells in athymic mice. Not many studies have been reported to establish the role of vitamin D analogs in preventing or retarding the metastasis of cancer cells to a distant organ, however a couple of reports as described above clearly hint that the selective analogs may be very influential against the cancer cell metastasis. Again, mechanistic studies have not been carried out in these models.

3. Clinical application of laboratory research

As described above vitamin D and its analogs have been examined for their efficacy in numerous in vitro and in vivo models to identify the most potent and yet non-toxic chemical forms of vitamin D. Many of these synthetic analogs have been evaluated in one or multiple models [16]. The reason for lack of analogs, which qualify for further evaluations, possibly in the clinical trials and subsequently as a chemopreventive or chemotherapeutic agent, is the toxicity to efficacy relationship. If a compound were toxic at a concentration which is effective in preventing experimental carcinogenesis or in suppressing cancer growth in experimental models then that analog would be of little value. This is one of the major reasons why 1α,25-(OH)2 D3, the active metabolite of vitamin D has not been employed in cancer prevention or treatment schedules. As discussed previously, the concentration, at which 1α,25 dihydroxy vitamin D3 is efficacious, is also sufficient to induce hypercalcemia in experimental animals. This was first observed by Koeffler and colleagues [54], by evaluating induction of cell differentiation of blast cells taken from the patients with acute myelogenous leukemia. Concentrations of 1μM induced cell differentiation but also was found to be toxic. The studies were extended by treating patients with myelodysplastc syndrome with 2 μg/day of 1,25(OH)2D3. Results also showed that 9 out of 18 patients developed hypercalcimia. In another study, safety and efficacy of both oral and topical treatments of 1α,25(OH)2D3 were evaluated for psoriasis. A study with 85 patients who received calcitriol (1α,25 (OH)2D3) showed that 88% of the patients showed some improvement in the disease. Among those responded to treatment, 26% showed complete protection from psoriasis. There was a significant increase in the calcium excretion, however renal function remained unaffected [55]. A similar mall clinical study was also carried out with 84 patients. The majority of the patients in this study responded to the topical treatment of 1.5 μg of calcitriol. No calcium metabolism abnormalities were observed. The study concluded that topical calcitriol was safe and effective for the treatment of psoriasis [56]. These early reports led to development of relatively nontoxic analogs of vitamin D. The agents that have received considerable attention include EB1089 (seocalcitol), MC903, RO24–5531, 1α-hydroxyvitamin D2, 25-hydroxyvitamin D3, 19-nor-1α,25-dihdyroxyvitamin D2 and 1α-hydroxyvitamin D5 [57,58,59]. These agents have been considered acceptable, based on the preclinical toxicity in animals under stringent experimental conditions. Early results with EB1089 confirmed low calcemic activity in a human maximum tolerated dose finding Phase I/II study [55]. Similar Phase I clinical trial with 36 patients with advanced breast cancer or colorectal cancers is also completed. The maximum tolerated dose of 16–24 nmole/m2 (total daily dose of 20–40nmole) for EB1089 was reported as compared to 2–4 nmole for 1,25-dihydroxyvitamin D3 [60]. Several reports in Japanese have also appeared for toxicity in experimental animals for MC903. Based on this clinical trials have been conducted for psoriasis using this analog, however the clinical trials for cancer patients have not been reported.

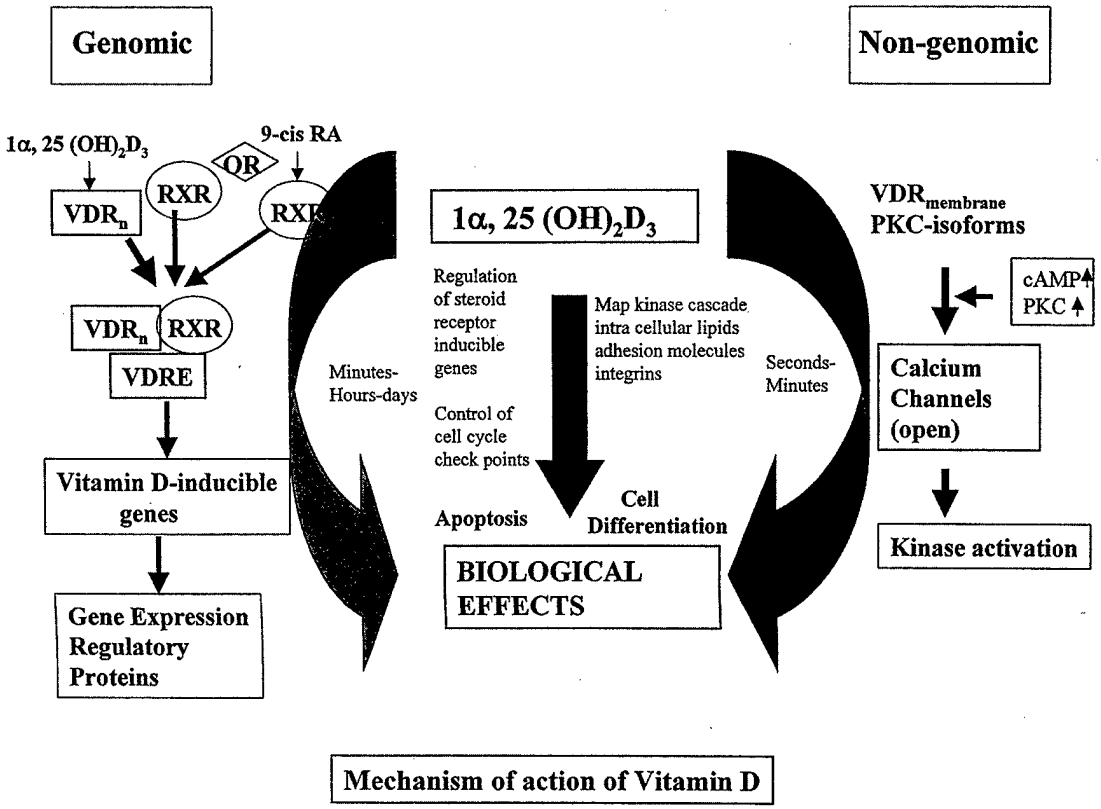

Fig. 4. Schematic diagram of potential mechanism of action of vitamin D. Vitamin D functions both via genomic and non-genomic pathways. Possible pathways of both these mediations of vitamin D action are shown in this diagram.

4. Mechanism of action of vitamin D and cancer

Vitamin D is classified as a steroid hormone [16]. The most unique feature for the steroid hormone has been its association with the specific nuclear receptors. The functional significance of the receptor-associated ligand is the initiation of a cascade of events involving signal transduction eventually leading to the biological function. As shown in Fig. 4, there are two distinct modes of action for vitamin D, one mediating vitamin D action via its binding with high affinity to its specific protein receptor (vitamin D receptor, VDR) and the second involving rapid functions using non-genomic membrane associated functions [61,62]. The non-genomic actions are generally very rapid, often the response could be within minutes as compared to genomic actions which may take longer period for the response. Vitamin D is unique in this respect since both these pathways have been well worked out and are supported by extensive evidence.

4.1. Genomic actions of vitamin D

Consistent with all other steroid hormones vitamin D mediates its action via VDR. Identification of VDR was initially made in chicken intestines by Haussler and Norman [63], followed by its preferential uptake by mammalian intestines and cell free binding of the cytosol to radioactive 1,25(OH)2D3, resulting in the saturable binding with a dissociation constant of 10–9 M. The sucrose density gradient studies of cytoplasmic VDR showed sedimentation of 3.5 S. Subsequently it was observed that the active metabolite associated with cytoplasmic VDR could bind to chromatin fractions [64]. Results have been reported over the years indicating localization of VDR in a variety of target organs and tissue types. These include digestive tract (esophagus and colon), mammary glands, prostate glands, lung alveolar cells brain neurons, connective tissues, fibroblasts, testes and ovaries as well as bone and osteoclasts. These results formed the basis for the establishment and future studies on VDR and its functional significance.

The VDR mRNA in human is a 4.8 kb whereas VDR is a 60 kD protein ranging from 400 to 27000 copies per cell yielding 10 to 100 femto (10–15)-moles/mg protein. Using anti-VDR antibody 9A7 cDNA, libraries derived from chicken intestine were screened in a viral expression system. Protein generated from a single clone from this screen reacted with the anti-VDR antibodies. Since then, VDR-cDNA has been sequenced using monoclonal antibody selection process [6]. Numerous reports collectively have concluded that there is a cluster of hormone receptors forming a family of steroid hormone receptor gene family [66]. Common structural motifs containing DNA binding do-

mains associated with regulatory domains are conserved during the evolution. These genes are under a direct control of transcription factors which regulate biological functions of cell proliferation, differentiation and death. As it is well established steroid hormone receptors are divided into five sections termed A through F. The segments A/B includes residues amino terminal to DNA binding domain, whereas C region contains highly conserved DNA binding domain. The ligand-binding domain at the carboxyl end is termed as either E or E/F region. The hinge on the other hand between C and E segments is termed as D region. While C region of the DNA binding domain is highly conserved, E section is the most flexible region. All regulatory controls reside in this region [67]. Within the ligand binding domain there are both homologies and structural differences among steroid hormones, which make them significantly different from other nuclear receptors including estrogen receptor, retinoic acid receptors, progesterone receptors, peroxisome proliferator activated receptor and thyroid hormone receptors [68]. In order for VDR to function, it needs to interact with vitamin D response element (VDRE) and bind to DNA. VDRE is a two identical hexanucleotide sequences separated by a spacer of 3 nucleotides. The spacer sequence is not conserved. Unlike estrogen receptors, this repeated sequence of two six-nucleotide segments, suggest that VDR must form a dimer for its action. Recent experiments have shown that VDR heterodimerizes with nuclear accessory factor (NAF) or retinoid X receptors (RXR). The natural metabolite 1,25(OH)2D3 transactivates VDRE in VDR positive cells but fails to show interaction in CV-11 (VDR-) cells. These results imply that the synthetic analogs transactivating VDR-VDRE interaction probably mediate their function via genomic pathway in a manner similar to dihydroxyvitamin D3 [69]. Results generated from our laboratory have shown that CV-1 cells transfected with VDR and VDRE when incubated with 1α(OH)D5 showed enhanced transactivation of VDR. Similarly, T47D and ZR75 ER+ VDR+ breast cancer cells express basal level of interaction with transient transfection of VDRE. However, co-transfection of VDR and VDRE significantly enhance the VDR-VDRE interaction when the cells are incubated with 1α(OH)D5 [29,70]. These results indicate that this analog of vitamin D mediates its action via genomic pathway.

Interactions among VDR and other receptors within the steroid receptor family have been a subject of a few investigations in recent years. Since the estrogen receptor positive and negative cells respond differently to vitamin D, in recent studies effects of 1,25-dihydroxyvitamin D3 on ER regulation have been investigated [71]. Results showed that all D-analogs evaluated, EB1089, KH-1060, R023–7553 down regulated ER levels when measured by western blot analysis as well as ligand binding assays. Moreover, this reduction was correlated with steady state levels of ER mRNA indicating direct down regulation of ER transcription by vitamin D analogs. In these studies, induction of progesterone receptors by estrogen was also reduced. More recently, in our laboratory we determined role of 1α-hydroxyvitamin D5 on cell cycle arrest and expression of progesterone receptors in BT474 cells. Results showed that cells were arrested in G1 phase accompanied with apoptosis down regulated estrogen inducible progesterone receptors [72]. Similar studies have also been conducted in prostate cancer cells to determine if vitamin D interacts with androgen receptors. Similar to ER+ breast cancer cells, androgen receptor (AR) positive LnCap cells respond better to vitamin D analogs compared to androgen resistant cells. Human glandular kallikerin (hK2) is an androgen regulated protein expressed in LnCap cells. Recent studies provide evidence for the role of vitamin D analogs for signaling pathways for androgen receptors [73].

The action of steroid hormone is regulated by various factors such as the receptor subtypes, regulation of hormone responsive gene promoters and the activation or suppression of function in response to steroid receptor complex. .Several cellular signaling pathways are involved in the regulation of gene expression by the steroid hormone receptors. The transcriptional activity of some hormone receptors is enhanced by protein kinase activators and growth factors. These proteins stimulate steroid receptor phosphorylation. These findings suggest that changes in steroid receptor phosphorylation are important in determining biological effects of these hormones and their receptors [74]. Alternatively, estrogen receptors could be activated by signals from tyrosine kinase-linked cell surface receptors. This process also involves phosphorylation of the kinases or the transcription factors [75]. Thus, either receptor phosphorylation or ligand bound receptor mediated phosphorylation of other factors is important for the receptor function. VDR like other receptors also get phosphorylated on the serine residues. The extent of phosphorylation is correlated to the extent of responsiveness of the cells to 1,25(OH)2D3 or calcitriol [76]. Furthermore, the phosphorylation is also correlated with VDR-VDRE interaction in transiently transfected cell system. These results suggest that 1,25(OH)2D3 mediated transcription may be dependent on VDR phosphorylation. Phosphorylation of human VDR has also been reported, however the extent of hVDR phosphorylation is significantly lower than the rat VDR phosphorylation [67]. In human, the majority of VDR phosphorylation is located at Serine 51. It has been fairly well established that the ser-51 phosphorylation is regulated by protein kinase C. The phosphorylatable residue at ser 51 is also observed for retinoic acid, thyroxin, and estrogen receptors. However, PKC-mediated phosphorylation is unique to VDR, the functional significance of PKC mediated phosphorylation is not conclusively demonstrated [77]. Both genomic activation by PKC-mediated phosphorylation and inhibition of VDR binding to DNA by this phosphorylation process has been reported [78]. In the later case, it is proposed that PKC dependent phosphorylation create a negative feed back loop that reduces availability of VDR for DNA binding. In addition to VDR phosphorylation by PKC, casein kinase II

and protein kinase A have also has been shown to phosphorylate VDR. Based on the working model for vitamin D action as proposed by Mark Hausler and colleagues [67], it is assumed that VDR resides in target cell nucleus are associated with DNA in a monomeric weak inactive confirmation. Upon binding with the ligand, VDR may become phosphorylated. Moreover, this complex allows dimerization of VDR with RXR. Phosphorylation of VDR and its heterodimerization allows inactivation of the repressor molecules. Haussler proposes that VDR-vitamin D complex dimerization with unliganded RXR makes it unresponsive to 9-cis retinoic acid. On the other hand, if RXR is preoccupied with its ligand then it can form homodimers as well as heterodimers. The homodimers may then disallow the vitamin D interaction with VDR and VDRE interaction. Needless to say that the understanding of genomic regulation of VDR mediated vitamin D function is far from complete. Yet, tremendous progress has been made to elucidate a delicate balance between receptors their interactions, phosphorylation of receptors and their regulatory proteins in order to understand molecular genomic mechanisms of vitamin D action.

4.2. Nongenomic rapid actions of vitamin D

While there is a wealth of information available and is constantly getting updated on the genomic actions of steroid receptors, not all actions of vitamin D can be explained by the genomic regulations. Anthony Norman and his colleagues have been studying the non-genomic action of vitamin D for the past 20+ years and have elegantly demonstrated that in addition to genomic actions of vitamin D there are rapid actions of the hormone largely mediated by membrane receptors of vitamin D and PKC [79,80]. The early studies demonstrated vitamin D mediated stimulation of calcium transport in chick duodenum called transcaltachia. Typically, the vitamin D induced initiation of responses in transcaltachia is not mediated by nuclear VDR directed signal transduction pathways. These responses occur within minutes unlike the genomic expression, which may take days prior to the modulation of endpoint markers. The rapid responses involve membrane receptors of vitamin D, and the pathways involved in induction of calcium channels leading to the exocytosis of calcium bearing vesicles from lysosomes. The ligand binding domain of the plasma binding protein, nuclear VDR and membrane VDR require unique shape of confirmationally flexible 1α,25(OH)2D3. The orientation and rigidity of the flexible side chain as well as the position of A ring in relation to C/D rings determine the vitamin D action. For example, the non-genomic responses including opening of the chloride channels, activation of PKC and MAP kinases require a planar 6-s-cis ligand shape which is recognized by the membrane-VDR as opposed to 6-s-trans bowl shaped 1α, 25(OH)2D3 required for nuclear-VDR interactions [81,82]. Involvement of nongenomic pathways for vitamin D action in carcinogenesis or prevention and therapy of cancer is not clearly defined, however in recent years increasing evidence for rapid effects of steroids that are incompatible with the classical genomic actions is accumulating. Norman and colleagues presented a Mannheim classification of nongenomic action at the first International meeting on the rapid actions of steroid hormones in Mannheim, Germany in 1998 [83]. According to this definition, the nongenomic action of steroid hormones is divided into six categories arbitrarily termed as A1, AIIa, AIIb, BI, BIIa, and BIIb. These differences in various types were according to the functional properties of the hormone. Type AI was classified as nongenomic direct action of steroids at high concentrations that does not require hormones. AIIa is direct action requiring classical receptor for example ERα induction of nitric oxide synthatase. Classification AII-b relates to a nongenomic rapid response transmitted by membrane receptors. Steroid hormones such as estradiol, mineralocorticoids, and vitamin D do function via membrane receptors. Finally, BII-b is the action of steroid hormone where the steroid functions as an agonist. There are several examples of such action in neuroendocrinological, function. However this would be out of scope for the current review and is not described in detail. The functional significance of BI and BII-a is not defined. Despite these developments, the rapid responses of vitamin D are not understood well in relation to the vitamin D action in cancer prevention or therapy.

In summary, in recent years role of synthetic analogs in the management of cancer patients has been extensively evaluated. Several hundred analogs of vitamin D have been synthesized and evaluated for their toxicity and efficacy in a variety of experimental models. To date only a few analogs have been considered for further development. Although it is generally accepted that the action of vitamin D is mediated via both genomic and non-genomic pathways, the major emphasis for the antiproliferative action of vitamin D analogs is placed on the VDR mediated action of the hormone. The VDR- breast and prostate cancer cells do not respond to vitamin D analogs. In steroid hormone receptor positive breast and prostate cancer cells the vitamin D acts by regulating steroid hormone inducible genes, whereas in the steroid receptor negative cells vitamin D induces cell differentiation. Understanding of the molecular mechanism of action of vitamin D will be crucial in generating more efficacious analogs of vitamin D in the prevention and treatment of cancer.

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References

[1] E. Mellanby, An experimental investigation on rickets, Lancet I 4985 (1919) 407–412.

[2] J.L. Napoli, M.A. Fivizzani, H.K. Schnoes, H.F. DeLuca, Synthesis of vitamin D5: its biological activity relative to D3 and D2, Arch. Biochem. Biophys. 197 (1979) 119–125.

[3] M.F. Holick, McCollum Award lecture Vitamin d-New horizons Am. J. Clin. Nutr. 60 (1994) 619–630.

[4] J.A. MacLaughlin, R.R. Anderson, M.F. Holick, Spectral character of sunlight modulates photosynthesis of previtamin D3 in the skin of poikilothermic animals, Science 216 (1982) 1001–1003.

[5] G.G. Schwartz, L.W. Whitlatch, T.C. Chen, B.L. Lokeshwar, M.F. Holick, Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25-hydroxyvitamin D3 Cancer Epidemiol, Biomarkers Prev. 7 (1998) 391–395.

[6] V. Tangpricha, J.N. Flanagan, L.W. Whitlatch, C.C. Tseng, T.C. Chen, P.R. Holt, M.S. Lipkin, M.F. Holick, 25-Hydroxyvitamin D-1-alpha-hydroxylase in normal and malignant colon tissue, Lancet 357 (2001) 1673–1674.

[7] H.S. Cross, P. Baeris, H. Hofer, M.G. Bischof, E. Bajna, S. Kriwanek, E. Bonner, M. Peterlik, 25-HydroxyvitaminD3–1(-hydroxyase and vitamin D receptor gene expression in human mucosa is elevated during early carcinogenesis, Steroids 66 (2001) 287–292.

[8] D. Hartwell, C. Hassenger, C. Christensen, Effect of vitamin D2 and D3 on the serum concentrations of 1,25 (OH)2D2 and 1,25(OH)2D3 in normal human subjects, Acta Endocrinol. 115 (1987) 378–384.

[9] H.S. Cross, M. Peterlik, G.S. Reddy, I. Schster, Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: expression of 25-hydroxyvitamin D3–1-alpha-hydroxylase activity and regulation of side-chain metabolism, J. Steroid Biochem. Mol. Biol. 62 (1997) 21–28.

[10] R.L. Horst, T.A. Reinhardt, Vitamin d metabolism, in: D. Feldman, F.H. Gloriewx, J.W. Pike (Eds.), Vitamin D, Academic Press, 1997, pp. 13–31.

[11] A. Nishimura, T. Shinki, C.H. Jin, Y. Ohayama, M. Noshiro, K. Okuda, T. Suda, Regulation of messenger RNA expression of 1,25 (OH)2D3–24-hydroxylase in rat osteoblasts 24 hydroxylase, Endocrinology 134 (1994) 1794–1799.

[12] H.L. Brenza, H.F. DeLuca, Regulation of 25-hydroxyvitamin D3 1alpha-hydroxylase gene expression by parathyroid hormone and 1,25-dihydroxyvitamin D3, Arch. Biochem. Biophys. 381 (1) (2000) 143–152.

[13] S.Y. James, M.A. Williams, S.M. Kelsey, A.C. Newland, K.W. Colston, The role of vitamin D derivatives and retinoids in the differentiation of human leukemia cells, Biochem. Pharmacol. 54 (1997) 625–634.

[14] S. Waxman, Differentiation therapy in acute myelogenous leukemia (non-APL), Leukemia 14 (3) (2000) 491–496.

[15] J.D. Roder, E. Stair, An overview of cholecalciferol toxicosis, Vet. Hum. Toxicol. 4 (1999) 344–348.

[16] R. Boullion, W.H. Okamura, A.W. Norman, Structure-function relationship in the vitamin D endocrine system, Endocrine Rev. 16 (1995) 200–257.

[17] A.W. Norman, X. Song, L. Zanello, C. Bula, W.H. Okamura, Rapid and genomic biological responses are mediate by different shapes of the agonist steroid hormone, 1alpha,25(OH)2vitamin D3, Steroids 64 (1999) 120–128.

[18] W.H. Park, J.G. Seol, E.S. Kim, J.M. Hyun, C.W. Jung, C.C. Lee, L. Binderup, H.P. Koeffler, B.K. Kim, Y.Y. Lee, Induction of apoptosis by vitamin D3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase, Br. J. Haematol. 109 (2000) 576–583.

[19] S. Suzuki, S. Takenoshita, H. Furukawa, A. Tsuchiya, Antineoplastic activity of 1,25(OH)2D3 and its analogue 22-oxacalcitriol against human anaplastic thyroid carcinoma cell lines in vitro, Int. J. Mol. Med. 4 (1999) 611–614.

[20] H. Matsumoto, Y. Iino, Y. Koibuchi, T. Andoh, Y. Horii, H. Takei, M. Horiguchi Maemura, T. Yokoe, Y. Morishita, Antitumor effect of 22-oxacalcitriol on estrogen receptor-negative MDA-MB-231 tumors in athymic mice, Oncol. Rep. 6 (1999) 349–352.

[21] C.M. Hansen, K.J. Hamberg, E. Binderup, L. Binderup, Secocalcitol (EB 1089): a vitamin D analogue of anti-cancer potential, background, design, synthesis, pre-clinical and clinical evaluation, Curr. Pharm. Des. 6 (2000) 803–828.

[22] E. Elstner, M. Linker-Israeli, J. Le, T. Umiel, P. Michl, J.W. Said, L. Binderup, J.C. Reed, H.P. Koeffler, Synergistic decrease of clonal proliferation, induction of differentiation, and apoptosis of acute promyelocytic leukemia cells after combined treatment with novel 20-epi vitamin D3 analogs and 9-cis retinoic acid, J. Clin. Invest. 99 (1997) 349–360.

[23] R.K. Wali, M. Bissonnette, S. Khare, J. Hart, M.D. Sitrin, T.A. Brasitus, 1 alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol, a noncalcemic analogue of 1 alpha,25-dihydroxyvitamin D3, inhibits azoxymethane-induced colonic tumorigenesis, Cancer Res. 55 (1995) 3050–3054.

[24] M.A. Anzano, J.M. Smith, M.R. Uskokovic, C.W. Peer, L.T. Mullen, J.J. Letterio., M.C. Welsh, M.W. Shrader, D.L. Logsdon, C.L. Driver, M.B. Sporn, 1 alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (RO24–5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat, Cancer Res. 54 (1994) 1653–1656.

[25] R.G. Mehta, R.M. Moriarty, R.R. Mehta, R. Penmasta, G. Lazzaro, A. Constantinou, L. Guo, Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1alpha-hydroxyvitamin D5, J. Natl. Cancer Inst. 89 (1997) 212–218.

[26] M.J. Campbell, A.F. Gombart, S.H. Kwok, S. Park, H.P. Koeffler, The anti-proliferative effects of 1alpha,25(OH)2D3 on breast and prostate cancer cells are associated with induction of BRCA1 gene expression, Oncogene 19 (2000) 5091–5097.

[27] R.R. Mehta, L. Bratescu, J.M. Graves, A. Green, R.G. Mehta, Differentiation of human breast carcinoma cells by a novel vitamin D analog: 1alpha-hydroxyvitamin D5, Int. J. Oncol. 16 (2000) 65–73.

[28] S. Swami, A.V. Krishnan, D. Feldman, 1alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells, Clin. Cancer Res. 6 (2000) 3371–3379.

[29] G. Lazzaro, A. Agadir, W. Qing, M. Poria, R.R. Mehta, R.M. Moriarty, T.K. Das Gupta, X.K. Zhang, R.G. Mehta, Induction of differentiation by 1alpha-hydroxyvitamin D(5) in T47D human breast cancer cells and its interaction with vitamin D receptors, Eur. J. Cancer 36 (2000) 780–786.

[30] J. Welsh, K. VanWeelden, L. Flanagan, I. Byrne, E. Nolan, C.J. Narvaez, The role of vitamin D3 and antiestrogens in modulating apoptosis of breast cancer cells and tumors, Subcell. Biochem. 30 (1998) 245–270.

[31] I.S. Mathiasen, K.W. Colston, L. Binderup, EB 1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells, J. Steroid Biochem. Mol. Biol. 46 (3) (1993) 365–371.

[32] R.G. Mehta, M.E. Hawthorne, V.E. Steele, Induction and prevention of carcinogen-induced precancerous lesions in mouse mammary gland organ culture, Methods Cell Sci. 19 (1997) 19–24.

[33] N.T. Telang, M.R. Banerjee, A.P. Iyer, A.B. Kundu, Neoplastic transformation of epithelial cells in whole mammary gland in organ culture, Proc. Natl. Acad. Sci. 76 (1979) 5886–5890.

[34] V. Sung, D. Feldman, 1,25-Dihydroxyvitamin D3 decreases human prostate cancer cell adhesion and migration, Mol. Cell. Endocrinol. 164 (2000) 133–143.

[35] G.G. Schwartz, M.H. Wang, M. Zang, R.K. Singh, G.P. Siegal, 1a, 25 Dihydroxyvitamin D (calcitriol) inghibits the invasiveness of human

prostate cancer cells, Cancer Epidemiol. Biomarkers Prev. 6 (1997) 727–732.

[36] A.M. Barreto, G.G. Schwartz, R. Woodruff, S.D. Cramer, 25-Hydroxyvitamin D3, the prohormone of 1,25-dihydroxyvitamin D3, inhibits the proliferation of primary prostatic epithelial cells, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 265–270.

[37] S.H. Zhuang, G.G. Scheartz, D. Cameron, K.L. Burnstein, Vitamin D receptor content and transcriptional activity do not predict antiproliferative effects of vitamin D in human prostate xancer cell lines, Mol. Cell. Endocrinol. 126 (1997) 83–90.

[38] S.Yamada, K. Yamamoto, H. Masuno, Structure-function analysis of vitamin D and VDR model, Curr. Pharm. Des. 6 (2000) 733–748.

[39] R. Vieth, Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety, Am. J. Clin. Nutr. 69 (1999) 842–856.

[40] J.M. Pettifor, D.D. Bikle, M. Cavaleros, D. Zachen, M.C. Kamdar, F.P. Ross, Serum levels of free 1,25-dihydroxyvitamin D in vitamin D toxicity, Ann. Intern. Med. 122 (1995) 511–513.

[41] R.C. Moon, R.G. Mehta, K.V.N. Rao, Retinoids and cancer in experimental animals, in: M.B. Sporn, A.B. Roberts, D.S. Goodman (Eds.), The Retinoids Biology, Chemistry and Medicine, Academic Press, 1994, pp. 573–596.

[42] R.G. Mehta, Experimental basis for the prevention of breast cancer, Eur. J. Cancer 36 (2000) 1275–1282.

[43] K.W. Colston, S.K. Chander, A.G. Mackay, R.C. Coombes, Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro, Biochem. Pharmacol. 44 (1992) 693–702.

[44] E.A. Jacobson, K.A. James, H.L. Newmark, K.K. Carroll, Effects of dietary fat, calcium, and vitamin D on growth and mammary tumorigenesis induced by 7,12-dimethylbenz(a)anthracene in female Sprague-Dawley rats, Cancer Res. 49 (22) (1989) 6300–6303.

[45] S.Y. James, E. Mercer, M. Brady, L. Binderup, K.W. Colston, EB1089, a synthetic analogue of vitamin D, induces apoptosis in breast cancer cells in vivo and in vitro, Br. J. Pharmacol. 125 (1998) 953–962.

[46] V. Mils, N. Basset-Seguin, J.P. Moles, J.J. Guilhou, 1,25-Dihydroxyvitamin D3 and its synthetic derivatives MC903 and EB1089 induce a partial tumoral phenotype reversal in a skin-equivalent system, J. Investig. Dermatol. Symp. Proc. 1 (1996) 87–93.

[47] R.G. Mehta, M.E. Hawthorne, L. Uselding, D. Albinescu, R. Moriarty, K. Christov, R. Mehta, Prevention of N-methyl-N-nitrosourea induced mammary carcinogenesis in rats by 1α-hydroxyvitamin D5, J. Natl. Cancer Inst. 92 (2000) 1836–1840.

[48] B.S. Reddy, The Fourth DeWitt S. Goodman lecture. Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 239–247.

[49] R.K. Wali, M. Bissonnette, S. Khare, J. Hart, M.D. Sitrin, T.A. Brasitus, 1 alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol, a noncalcemic analogue of 1 alpha,25-dihydroxyvitamin D3, inhibits azoxymethane-induced colonic tumorigenesis, Cancer Res. 55 (14) (1995) 3050–3054.

[50] T. Otoshi, H. Iwata, M. Kitano, Y. Nishizawa, H. Morii, Y. Yano, S. Otani, S. Fukushima, Inhibition of intestinal tumor development in rat multi-organ carcinogenesis and aberrant crypt foci in rat colon carcinogenesis by 22-oxa-calcitriol, a synthetic analogue of 1 alpha, 25-dihydroxyvitamin D3, Carcinogenesis 16 (9) (1995) 2091–2097.

[51] R.R. Mehta, J.M. Graves, G.D. Hart, A. Shilkaitis, T.K. Das Gupta, Growth and metastasis of human breast carcinoma with matrigel in athymic mice, Breast Cancer Res. Treat. 25 (1993) 65–71.

[52] R.R. Buras, L.M. Schumaker, F. Davoodi, R.V. Brenner, M. Shabahang, R.J. Nauta, S.R. Evans, Vitamin D receptors in breast cancer cells, Breast Cancer Res. Treat. 31 (1994) 191–202.

[53] B.L. Lokeshwar, G.G. Schwartz, M.G. Selzer, K.L. Burnstein, S.H. Zhuang, N.L. Block, L. Binderup, Inhibition of prostate cancer metastasis in vivo: a comparison of 1,23-dihydroxyvitamin D (calcitriol) and EB1089, Cancer Epidemiol Biomarkers Prev. 8 (1999) 241–248.

[54] H.P. Koeffler, K. Hirji, L. Itri, 1,25-Dihydroxyvitamin D3: in vivo and in vitro effects on human preleukemic and leukemic cells, Cancer Treat. Rep. 69 (1985) 1399–1407.

[55] A. Perez, R. Raab, T.C. Chen, A.Turner, M.F. Holick, Safety and efficacy of oral calcitriol (1,25-dihydroxyvitamin D3) for the treatment of psoriasis, Br. J. Dermatol. 134 (1996) 1070–1078.

[56] A. Perez, T.C. Chen, A. Turner, R. Raab, J. Bhawan, P. Poche, M.F. Holick, Efficacy and safety of topical calcitriol (1,25-dihydroxyvitamin D3) for the treatment of psoriasis, Br. J. Dermatol. 134 (1996) 238–246.

[57] C.M. Hansen, K.J. Hamberg, E. Binderup, L. Binderup, Seocalcitol (EB 1089): a vitamin D analogue of anti-cancer potential. Background, design, synthesis, pre-clinical and clinical evaluation, Curr. Pharm. Des. 6 (2000) 803–828.

[58] T.C. Chen, G.G. Schwartz, K.L. Burnstein, B.L. Lokeshwar, M.F. Holick, The in vitro evaluation of 25-hydroxyvitamin D3 and 19-nor-1alpha,25-dihydroxyvitamin D2 as therapeutic agents for prostate cancer, Clin. Cancer Res. 6 (2000) 901–908.

[59] W.D. Johnson, R.R. Mehta, R.M. Moriarty, R.G. Mehta, T.K. Das Gupta, D.L. McCormick, Preclinical toxicity of 1a-hydroxyvitamin D5 in rats and dogs, Proc. Am. Assoc. Cancer Res. 42 (2001) 933.

[60] T. Guilliford, J. English, K. Colston, P. Sprogel, R.C. Coombes, A Phase I study of EB 1089, a vitamin D analog, Proc. Am. Assoc. Cancer Res. 37 (1996) 1130.

[61] E. Falkenstein, A.W. Norman, M. Wehling, Mannheim classification of nongenomically initiated (rapid) steroid action(s), J. Clin. Endocrinol. Metab. 85 (2000) 2072–2075.

[62] G. Jones, S.A. Strugnell, H.F. DeLuca, Current understanding of the molecular actions of vitamin D, Physiol. Rev. 78 (1998) 1193–1231.

[63] M.R. Haussler, A.W. Norman, Chromosomal receptors for a vitamin D metabolite, Proc. Natl. Acad. Sci. USA 62 (1969) 155–162.

[64] V. Boonyaratanakornkit, V. Melvin, P. Prendergast, M. Altmann, L. Ronfani, M.E. Bianchi, L. Taraseviciene, S.K. Nordeen, E.A. Allegretto, D.P. Edwards, High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells, Mol. Cell Biol. 18 (1998) 4471–4487.

[65] C. Rachez, L.P. Freedman, Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions, Gene 246 (4) (2000) 9–21.

[66] G.K. Whitfield, P.W. Jurutka, C.A. Haussler, M.R. Haussler, Steroid hormone receptors: evolution, ligands, and molecular basis of biologic function, J. Cell Biochem. 32 (1999) 110–122.

[67] A.R. Baker, D.P. McDonnell, M.R. Hughes, T.M. Crisp, D.J. Mengelsdorf, M.R. Haussler, J. Shine, J.W. Pike, B.W. Omalley, Molecular cloning and expression of human vitamin D receptor complementary DNA: structural homology with thyroid hormone receptors, Proc. Natl. Acad. Sci. 85 (1988) 294–298.

[68] X. Zhang, M. Jeyakumar, M.K. Bagchi, Ligand-dependent cross-talk between steroid and thyroid hormone receptors, evidence for common transcriptional coactivator(s), J. Biol. Chem. 271 (1996) 14825–14833.

[69] M.R. Haussler, P.W. Jurutka, J. Hseih, P.D. Thompson, C.A. Haussler, S.H. Selznick, L.S. Remus, G.K. Whitfield, Nuclear vitamin d receptor: structure function phosphorylation and control of gene transcription, in: D. Feldman, F. Glorieux, J.W. Pike (Eds.), Vitamin D, Academic Press, 1997, pp. 149–177.

[70] A. Agadir, G. Lazzaro, Y. Zheng, X.K. Zhang, R.G. Mehta, Resistance of HBL100 human breast epithelial cells to vitamin D action, Carcinogenesis 20, 577–582.

[71] A. Stoica, M. Saceda, A. Fakhro, H.B. Solomon, B.D. Fenster, M.B. Martin, Regulation of estrogen alpha gene expression by 1,25 dihydroxyvitamin D in MCF-7 cells, J. Cell Biochem. 15 (1999) 640–651.

[72] E.A. Hussain, K.P. Bhat, R.R. Mehta, R.G. Mehta, 1α-Hydroxyvitamin D5 induces apoptosis and cell cycle arrest in BT474 breast cancer cells, Proc. Am. Assoc. Cancer Res. 42 (2001) 209.

[73] S.H. Mitchell, P.E. Murtha, S. Zhang, W. Zhu, C.Y. Young, An androgen response element mediates LnCap cell dependent on androgen induction of hK2 gene, Mol. Cell Endocrinol. 168 (2000) 89–99.

[74] B.S. Katzenallenbogen, Mechanisms of action of cross talk between estrogen receptor and progesterone receptor pathways, J. Soc. Gynecol. Investig. 7 (2000) S33–S37.

[75] R. Duan, W. Xie, R.C. Burghardt, S. Safe, Estrogen receptor mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of ELK-1, J. Biol. Chem. 276 (2001) 11590–11598.

[76] H.M. Darwish, J.K. Burmester, V.E. Moss, H.F. DeLuca, Phosphorylation is involved in transcriptional activation by the 1,25 dihydroxyvitamin D3 receptor, Biochem. Biophys. Acta 1167 (1993) 29–36.

[77] J. Hseih, P.W. Jurutka, S. Nakajima, M.A. Galligan, C.A. Haussler, Y. Shimizu, N. Shimizu, G.K. Whitefeld, M.R. Haussler, Phosphorylation of the human vitamin d receptor by protein kinase C., biochemical and functional evaluations of the serine 51 recognition site, J. Biol. Chem. 268 (1993) 15118–15126.

[78] J.P. VanLeeuwen, J.C. Birkenhager, G.J. van den Bemd, C.J. Buurman, A. Stall, M.P. Bos, H.A.P. Pols, Evidence for the functional involvement of protein kinase C in the action of 1,25 dihydroxyvitamin D3 in bone, J. Biol. Chem. 267 (1992) 12562–12569.

[79] I. Nemere, A.W. Norman, Studies on the mode of action of calciferol rapid actions of 1,25 dihydroxyvitamin D3 on calcium transport in purfused chick duodenum, J. Bone Miner. Res. 2 (1984) 99–107.

[80] R.K. Wali, C.L. Baum, M.D. Sitrin, M.J. Bolt, P.K. Dudeja, T.A. Brasitus, Effect of vitamin D status on the rapid actions of 1,25-dihydroxycholecalciferol in rat colonic membranes, Am. J. Physiol. 262 (1992) G945–953.

[81] A.W. Norman, S. Ishuzuka, W.H. Okamura, Ligands for the vitamin D endocrine system: different shapes function as agonists and antagonists for genomic and rapid response receptors or as a ligand for the plasma vitamin d binding protein, J. Steroid Biochem. Molec. Biol. 76 (2001) 49–59.

[82] A.W. Norman, H.L. Henry, J.E. Bishop, X.D. Song, C. Bula, W.H. Okamura, Different shapes of the steroid hormone 1(,25(OH)2-vitamin D3 act as agonists for two different receptors in the vitamin D endocrine system to mediate genomic and rapid responses, Steroids 66 (2001) 147–158.

[83] E. Falkenstein, A.W. Norman, M. Wehling, Mannheim classification of nongenomically initiated (rapid) steroid action(s), J. Clin. Endocrinol Metabol. 85 (2000) 2072–2075.

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

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ABSTRACT

Numerous analogs of vitamin D have been synthesized in recent years with the hope of generating a compound that retains the anticarcinogenic activity of vitamin D without causing any toxicity. We synthesized such an analog, 1α-hydroxy-24-ethylcholecalciferol [1α-hydroxyvitamin D5 or 1α(OH)D_5], and showed that it was tolerated by rats and mice at a much higher dose than 1α,25 dihydroxy cholecalciferol [1α,25$(OH)_2$D3]. 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 to 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 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.

INTRODUCTION

Conceptually, chemoprevention of cancer can be defined as an intervention in the carcinogenic process by either a naturally derived or a synthetic compound. An agent that blocks, arrests, or reverses the progression of cancer can be termed a chemopreventive agent (1,2). In practice, this can best be achieved by the dietary administration of chemical agents, which can enhance the physiological processes that protect the organism against the development of malignancy. Current understanding of progression of a normal cell to a transformed cancer cell is summarized in Figure 1. Under experimental conditions, a normal cell could be transformed to an initiated cell in response to carcinogenic or mutagenic stimuli. Although the initiated cells have 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-initiating 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. (5,6,7). In this report, we have summarized the chemopreventive properties of a newly evaluated vitamin D analog, 1-α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D_5].

It has been well established that the active metabolite of vitamin D 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3] is a steroid hormone and it exhibits potent cell-

differentiating properties in leukemia cells as well as other cancer cells of epithelial origin (8,9). The antiproliferative and differentiation-inducing effects of $1,25(OH)_2D3$ 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 $1\alpha,25(OH)_2D3$ are cytotoxic (11). The effective growth inhibitory concentration of $1\alpha,25(OH)_2D3$ 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 (Figure 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 vitamins D2, D3, D4, D5, and D6, had suggested that vitamin D5 was the least toxic of the D series of compounds (16). In order to generate an effective but non-calcemic and non-toxic vitamin D analog, we synthesized $1\alpha(OH)D_5$ (17). The structure of $1\alpha(OH)D_5$ is shown in Figure 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic pathway involves its association with high-affinity specific vitamin D receptor (VDR) that belongs to the steroid receptor superfamily of ligand-activated transcription factors

(18,19,20). This is consistent with the well-known mode of action of the steroid hormones. The VDR has been identified in a variety of tissues such as breast, prostate, liver, fibroblasts, colon, and lungs (21), in addition to the previously known target organs that included intestine, kidney, and bone.

The VDR mRNA is about 4.6 kb, which translates to a 50-kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell, yielding 10 to 100 femtomoles per mg of total protein. In order for VDR to function, it needs to bind specific DNA sequences and interact with vitamin D response elements (VDRE) (22). The natural metabolite $1\alpha,25(OH)_2D3$ transactivates VDRE in VDR^+ cells but fails to show interaction in VDR^- cells. Hence, vitamin D analogs that are able to transactivate VDR-VDRE are mainly mediating their action via genomic pathways. Non-genomic vitamin D actions have been studied mostly in relation to calcium and phosphorus metabolism, and to a lesser extent with respect to chemoprevention. The rapid responses involve a putative membrane receptor of vitamin D that signals to modulate calcium channel activity in a cell. This may lead to exocytosis of calcium-bearing vesicles from lysosomes. The non-genomic pathway for vitamin D action has been extensively reviewed elsewhere (23,24). For this article, we have listed the chemopreventive properties and possible mode of action of $1\alpha(OH)D_5$.

MATERIALS AND METHODS

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^+ VDR^+ breast cancer cell lines BT474 and MCF7; and (3) ER^- PR^- VDR^- breast cancer cell lines MDA-MB-231 and MDA-MB-435 cell lines.

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 Figure 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Weymouth's 752/1 medium under 95% O_2 and 5% CO_2 at 37°C. The glands respond to growth-promoting hormones insulin, prolactin, aldosterone, and hydrocortisone and differentiate into distinct alveolar structures. Exposure of glands to 7,12 dimethylbenz(a)anthracene (DMBA) for 24 hours on Day 3 of culture results in the development of precancerous mammary alveolar lesions (MAL). If the growth-promoting medium contains estrogen and progesterone instead of aldosterone and hydrocortisone, the glands develop mammary ductal lesions (MDL) with DMBA treatment (26). We performed a dose response study to compare the effects of $1\alpha(OH)D_5$ 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.

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 minutes. 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.

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 40x objective lens using a flourescein filter. Approximately 100 cells were counted on each slide to assess the proportion of cells undergoing apoptosis.

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 Figure 4. Briefly, 100-day-old female Sprague/Dawley rats 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.

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.

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.

RESULTS AND DISCUSSION

Synthesis and Toxicity of $1\alpha(OH)D_5$: Nearly 300 analogs of $1,25(OH)_2D3$ 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) and are relatively nontoxic at effective concentrations in experimental models. The hexafluoro analog of $1,25(OH)_2D3$, R024-5531, has no calcemic activity, while other analogs do express dose-related calcemia (33,34). Since it had been reported previously that vitamin D5 is the least toxic series of vitamin D compounds, we synthesized $1\alpha(OH)D_5$ 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 $1,25(OH)_2D3$ 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 $1,25(OH)_2D3$ 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 $1,25\ (OH)_2D3$ (0.8 to 12.8 μg/kg diet) and $1\alpha(OH)D_5$ (6.4 to 50 μg/kg diet) for two months. Calcium concentration was

increased by 1,25 $(OH)_2D3$ 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.

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 Figure 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 $1,25(OH)_2D3$. The most effective dose of $1,25(OH)_2D3$ in

suppressing >60% incidence of MAL is 10^{-7}M, while 1α(OH)D_5 is equally effective at 10^{-6}M without showing cytotoxicity. We also evaluated 1α(OH)D_5 effects in the MDL model (25). The results are summarized in Figure 5. We found 1α(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 1α(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, 1α(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 1α(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 1α(OH)D_5, ranging from 1 to 100 μg/kg diet for six weeks. The animals did not show any adverse effects at any concentration of 1α(OH)D_5, while the natural hormone was toxic at 3.5 μg/kg diet.

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

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^- VDR^- highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both $1,25(OH)_2D3$ 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^+ 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. As shown in Figure 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 $ER^+PgR^+VDR^+$ MCF-7, ZR75/1, and BT474 cells or $ER^-PgR^-VDR^+$ BCA-4 cells responded to 12.5 g $1\alpha(OH)D_5$/ 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 (Figure 7). Contrarily, $1\alpha(OH)D_5$ showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Figure 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 hours 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 condensed chromatin and reduced Ki 67 expression after 72-hour incubation with $1\alpha(OH)D_5$ (Mehta, unpublished data). Taken together, these results indicate that, in human breast epithelial tissues, $1\alpha(OH)D_5$ is selective for its effects on pre-cancerous or cancer cells but shows no effect normal breast epithelial cell growth.

Mechanism of $1\alpha(OH)D_5$ Action: The effects of $1\alpha(OH)D_5$ 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 $1\alpha(OH)D_5$ and its efficacy as an anti-proliferative agent. We had reported that, in ER^+ PgR^+ breast cancer cells, $1\alpha(OH)D_5$ 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 $1\alpha(OH)D_5$ depended not only on expression of VDR but also on the expression of ER and ER-inducible genes such as PgR.

The effects of $1\alpha(OH)D_5$ on cell cycle were determined using breast cancer cells. The BT474 cells were treated with 1 μM $1\alpha(OH)D_5$ for various time points and processed for FACS analysis as described in the Materials and Methods section. 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 $1,25(OH)_2D3$ or $1\alpha(OH)D_5$ for 72 hours and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Figure 8 shows that BT474 cells underwent apoptosis with $1\alpha(OH)D_5$ 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 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.

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REFERENCES

1. M.B. Sporn, K.W. Hong. Recent advances in chemoprevention of cancer, *Science*. 278 (1997) 1073-1077.

2. L.W. Wattenberg. Inhibition of tumorigenesis in animals. In: Principles of Chemoprevention IARC Handbook on Chemoprevention, 1996, pp 151-158.

3. G.J. Kelloff, C.W. Boone, W.E. Malone, V.E. Steele. Recent results in preclinical and clinical drug development of chemoppreventive agents at the National Cancer Institute. In: L. Wattenberg, M. Lipkin, C.W. Boone, G.J. Kelloff (Eds.), Cancer Chemoprevention, Boca Raton, Florida, CRC Press, 1992, pp. 42-54.

4. S. DeFlora, A. Izzotti, F. D'Agostine, R.M. Balausky, D. Noonan, A. Altsin. Multiple points of intervention in the prevention of cancer and other mutation-related diseases, *Mutat Res*. 480-481 (2001) 9-22.

5. G.J. Kelloff, C.W. Boone, J.A. Crowell, V.E. Steele, R. Lubet, C.C. Sigman. Chemopreventive drug development: perspectives and progress, *Cancer Epidemiol Biomarkers Rev*. 3 (1994) 85-98.

6. S.M. Rosenbaum Smith, M.P. Osborne. Breast cancer chemoprevention, *Am J Surg*. 180 (2000) 249-251.

7. G.J. Kelloff, C.W. Boone, J.A. Crowell, V.E. Steele et al. New agents for cancer chemoprevention, *J Cell Biochem*. Suppl 26 (1996) 1-28.

8. S. Y. James, M.A. Williams, S.M. Kessey, A.C. Newland, K.W. Colston. The role of vitamin D derivatives and retinoids in the differentiation of human leukemia cells, *Biochem Pharmacol*. 54 (1997) 625-34.

9. S. Waxman. Differentiation therapy in acute myelogenous leukemia (non-APL), *Leukemia.* 14 (2000) 491-6.

10. S. Christakos. Vitamin D in breast cancer, Adv Exp Med Biol. 364 (1994) 115-118.

11. J.D. Roder, E. Stair. An overview of cholecalciferol toxicosis, *Vet Human Toxicol.* 4 (1999) 344-348.

12. R. Vieth. Vitamin D supplementation, 25(OH)D3 concentration and safety, Am J Clin Nutr. 69 (1999) 842-856.

13. M.J. Calverley. Novel side chain analogs of 1α,25-dihydroxyvitamin D3: design and synthesis of the 21,24-methano derivatives, *Steroids.* 66 (2001) 249-255.

14. R. Boullion, W.H. Okamura, A.W. Norman. Structure-function relationship in the vitamin D endocrine system, *Endocrine Rev.* 16 (1995) 200-257.

15. A.W. Norman. The vitamin D endocrine system: manipulation of structure function relationship to provide opportunities for development of new cancer chemopreventive and immunosuppressive agents, *J Cell Biochem.* Suppl 22 (1995) 218-225.

16. J.L. Napoli, M.A. Fivizzani, H.K. Schnoes, H.F. DeLuca. Synthesis of vitamin D5: its biological activity relative to vitamins D3 and D2, *Arch Biochem Biophys.* 197 (1979) 119-125.

17. R.G. Mehta, R.M. Moriarty, R.R. Mehta, R. Penmasta, G. Lazzaro, A. Constantinou, L. Guo. Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1α-hydroxyvitamin D5, *J Natl Cancer Inst.* 89 (1997) 212-218.

18. M.R. Haussler. Vitamin D Receptors: nature and function, *Annu Rev Nutr.* 6 (1986) 527-62.

19. R.L. Horst, T.A. Reinhardt. Vitamin D metabolism. In: D. Feldman, F.H. Gloriewx, J.W. Pike (Eds), Vitamin D, Academic Press, pp 13-31.

20. C. Rachez, L.P. Freedman. Mechanism of gene regulation by VDR: a network of coactivator interactions, *Genes*. 246 (2000) 9-21.

21. J.W. Pike. Vitamin D3 Receptors: structure and function in transcription, *Annu Rev Nutr*. 11 (1991) 189-216.

22. G. Jones, S.A. Struguell, H.F. DeLuca. Current understanding of the molecular actions of vitamin D, *Physiol Rev*. 78 (1998) 1193-1231.

23. A.W. Norman, X. Song, L. Zanello, C. Bula, W.H. Okamura. Rapid and genomic biological responses are mediated by different shapes of the agonist steroid hormone, 1α,25(OH)$_2$vitamin D3, *Steroids*. 64 (1999) 120-128.

24. E. Falkenstein, A.W. Norman, M. Wehling. Mannheim classification of non-genomically initiated rapid steroid actions, *J Clin Endocrinol Metab*. 85 (2000) 2072-2075.

25. R.G. Mehta, M.E. Hawthorne, V.E. Steele. Induction and prevention of carcinogen-induced precancerous lesions in mouse mammary gland organ culture, *Methods in Cell Science*. 19 (1997) 19-24.

26. R.G. Mehta, P.L. Bhat, M.E. Hawthorne, et al. Induction of atypical ductal hyperplasia in mouse mammary organ culture, *J Natl Cancer Inst*. 93 (2001) 1103-1106.

27. L.L. Vindeløv, I.J. Christensen, N.I. Nissen. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis, *Cytometry*. 3 (1983) 323-327.

28. R.G. Mehta, M.E. Hawthorne, L. Uselding, *et al*. Prevention of MNU-induced mammary carcinogenesis in rats by 1α(OH)D5, *J Natl Cancer Inst*. 92 (2000) 1836-1840.

29. C.M. Hansen, K.J. Hamberg, E. Binderup, L. Binderup. Secocalcitol (EB1089), a vitamin D analog of anticancer potential. *Curr Pharm Des*. 6 (2000) 803-828.

30. S.P. Xie, S.Y. James, K.W. Colston. Vitamin D derivative inhibit the mitogenic effects of IGF-1 on MCF-7 human breast cancer cells, *J Endocrinol.* 154 (1997) 495-504.

31. M.A. Anzano, J.M. Smith, M.R. Uskokovic, C.W. Peer, L.T. Muller, *et al.* 1α,25(OH)2-16-ene-23-ene-26,27-hexafluoro-cholecaciferol (R024-5531), a new deltanoid for prevention of breast cancer in rats, *Cancer Res.* 54 (1994) 1653-1656.

32. H. Matsumoto, Y. Iino, Y. Korbuchi, *et al.* Anti-tumor effect of 22-Oxacalcitriol on estrogen receptor negative MDA-MB-231 tumors in athymic mice, *Oncol Rep.* 6 (1999) 349-352.

33. K.Z. Guyton, T.W. Kensler, G.H. Posner. Cancer chemprevention using natural vitamin D and synthetic analogs, *Annu Rev Pharmacol Toxicol.* 41 (2001) 421-42.

34. T.W. Kensler, P.M. Dolan, S.J. Gange, J.K. Lee, O.Wang, G.H. Posner. Conceptually new deltanoids (vitamin D analogs) inhibit multistage skin tumorigenesis, *Carcinogenesis.* 21 (2000) 1341-1345.

35. R.R. Mehta, L. Bratesca, J.M. Graves, A. Green, R.G. Mehta. Differentiation of human breast carcinoma cells by a novel vitamin D analog, *Int J Oncol.* 16 (2000) 65-73.

36. J. Welsh, K. VanWeelden, L. Flanagan, I. Byrne, E. Nolan, C.J. Narvaez. The role of vitamin D3 and antiestrogens in modulating apoptosis of breast cancer cells and tumors, *Subcell Biochem.* 30 (1998) 245-270.

37 R.G. Mehta, R.R. Mehta. Vitamin D and Cancer, *J Nutr Biochem.* 2002 (In press).

38. S.Y. James, A.G. Mackay, K.W. Colston. Effects of 1,25(OH)2D3 and its analogs on induction of apoptosis in breast cancer cells, J Steroid Biochem Molec Biol. 58 (1996) 395-401.

Figure Legends

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

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

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

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

Figure 5. Effect of $1\alpha(OH)D5$ on mouse mammary organ culture (MMOC). The glands were incubated with 1 μM $1\alpha(OH)D5$ 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.

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

experiment was repeated twice and differences between the mean were assessed using student's *t* test.

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

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

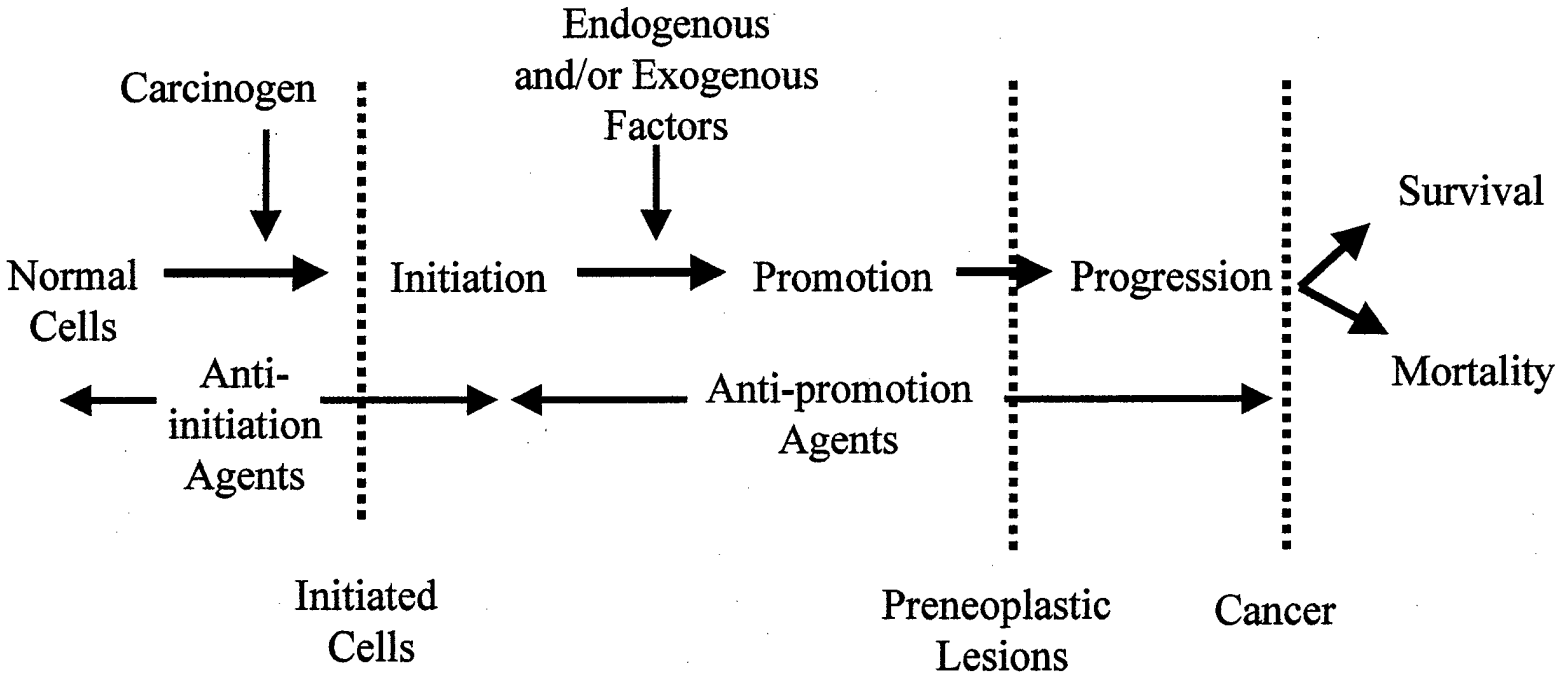

Carcinogen
Endogenous
and/or Exogenous
Factors
Survival
Normal
Cells
Initiation
Promotion
Progression
Mortality
Anti-
initiation
Agents
Anti-promotion
Agents
Initiated
Cells
Preneoplastic
Lesions
Cancer

Figure 1

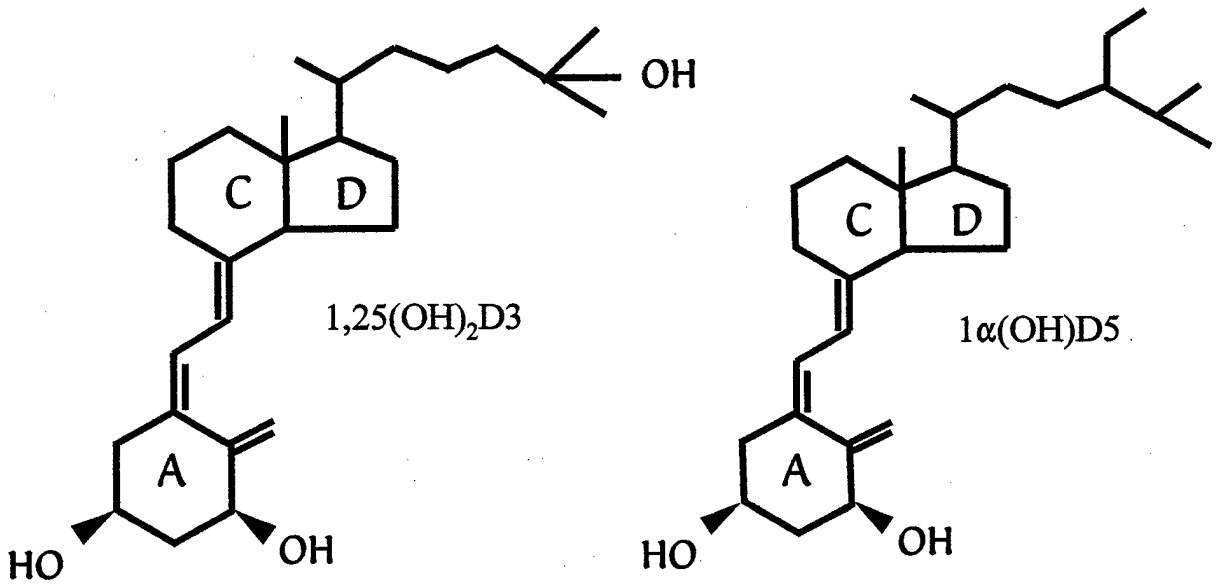
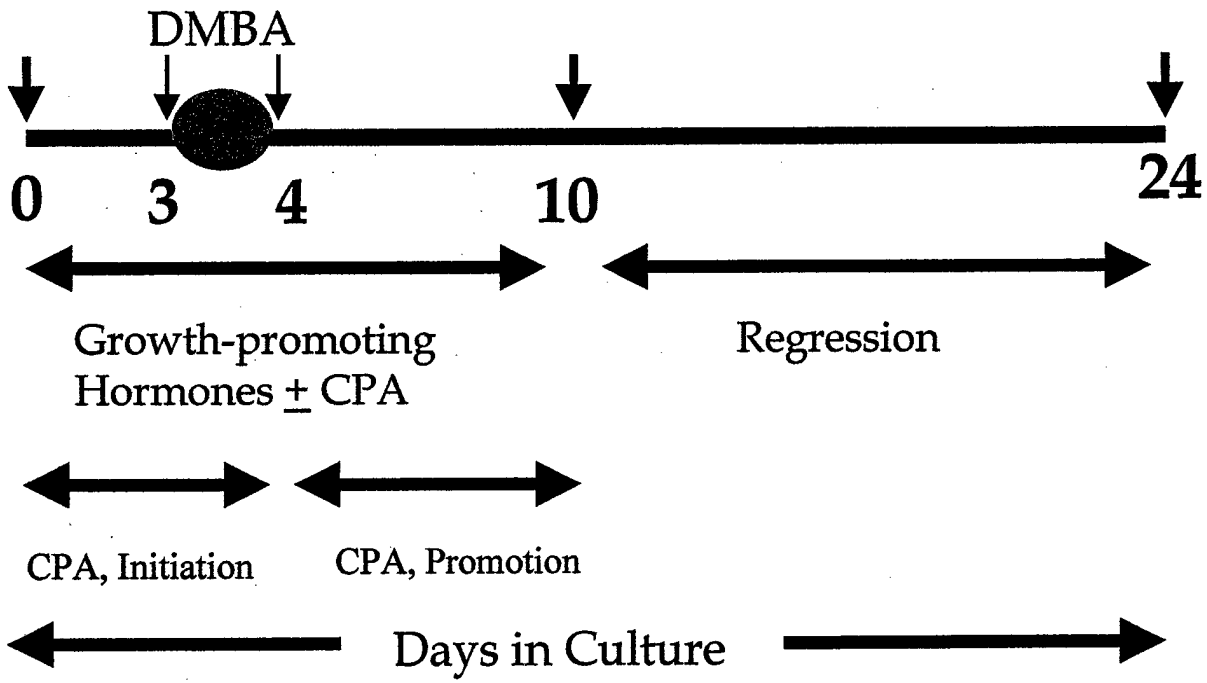


Figure 2



DMBA + IPAF = Alveolar Lesions (MAL) E_2 Independent
DMBA + IPEPg = Ductal Lesions (MDL) E_2 Dependent

Figure 3

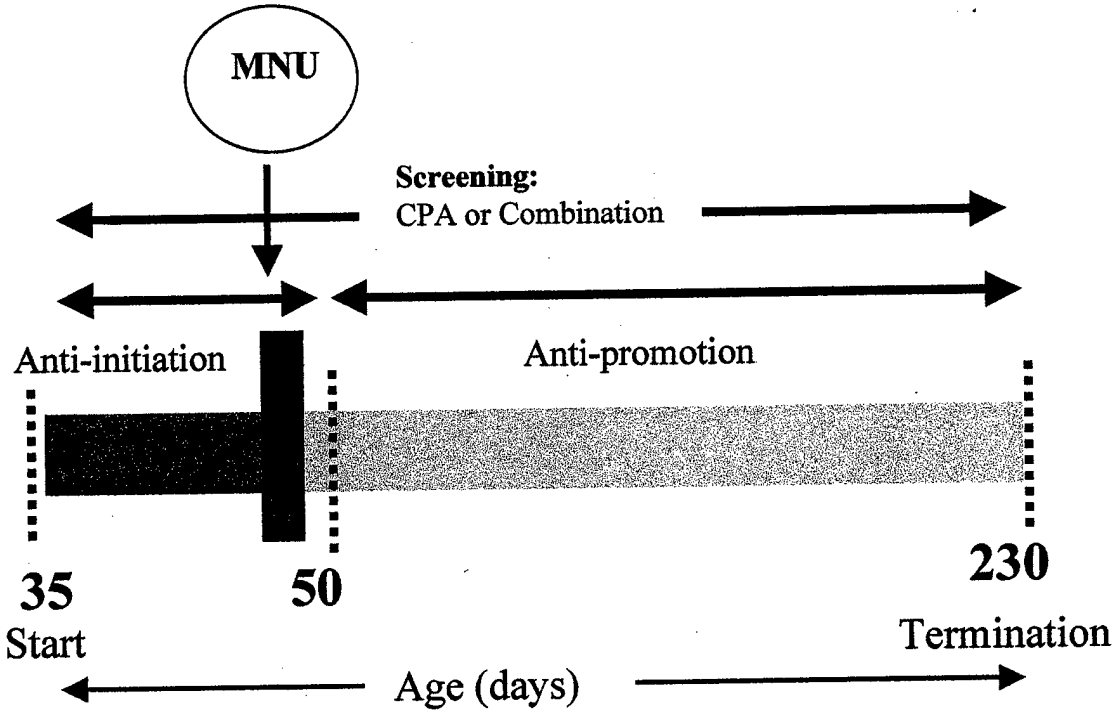

MNU
Screening:
CPA or Combination
Anti-initiation
Anti-promotion
35
Start
50
230
Termination
Age (days)

Figure 4

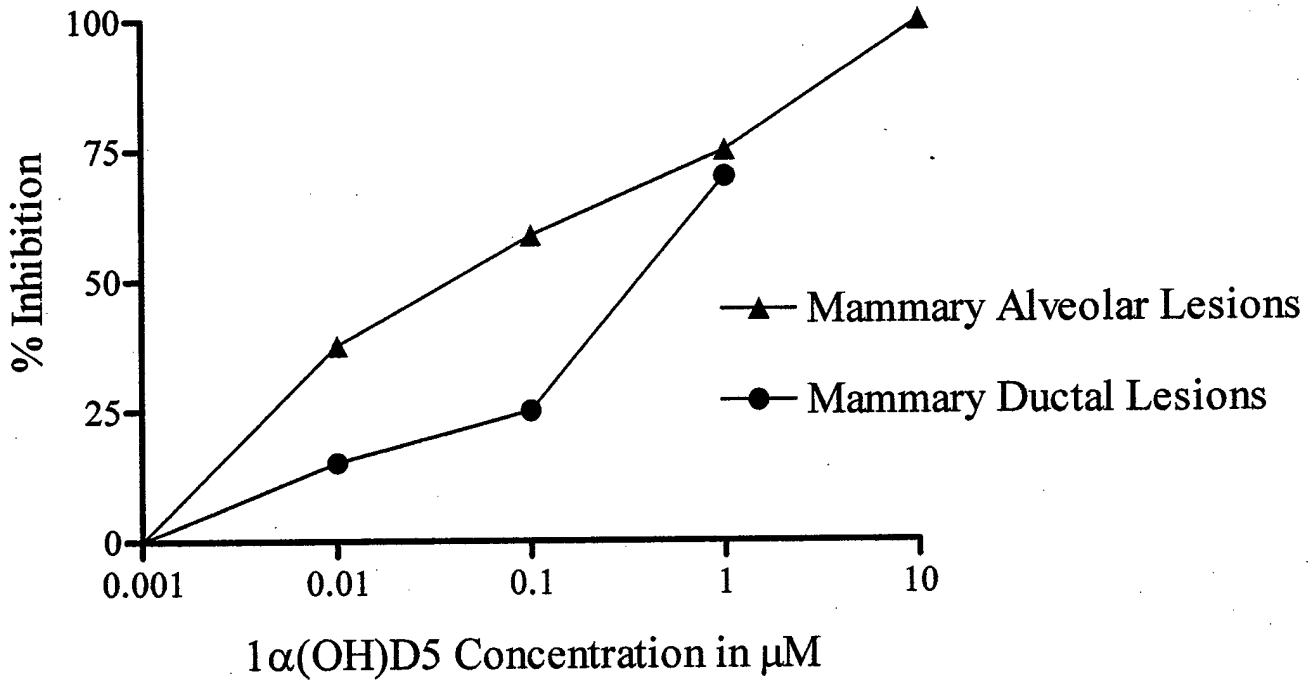

100
75
50
25
0
% Inhibition
0.001
0.01
0.1
1
10
1α(OH)D5 Concentration in μM
Mammary Alveolar Lesions
Mammary Ductal Lesions

Figure 5

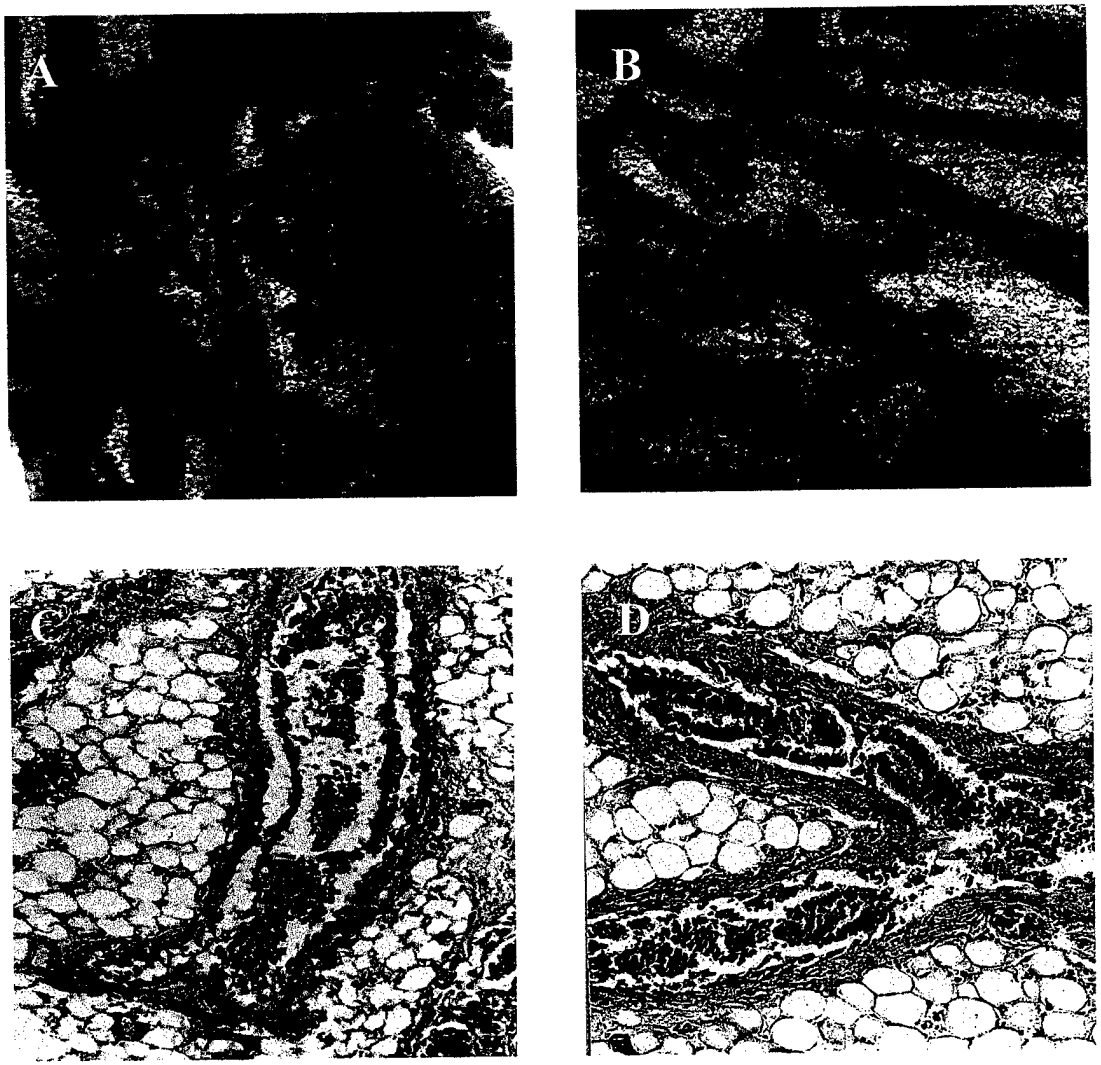


Figure 6

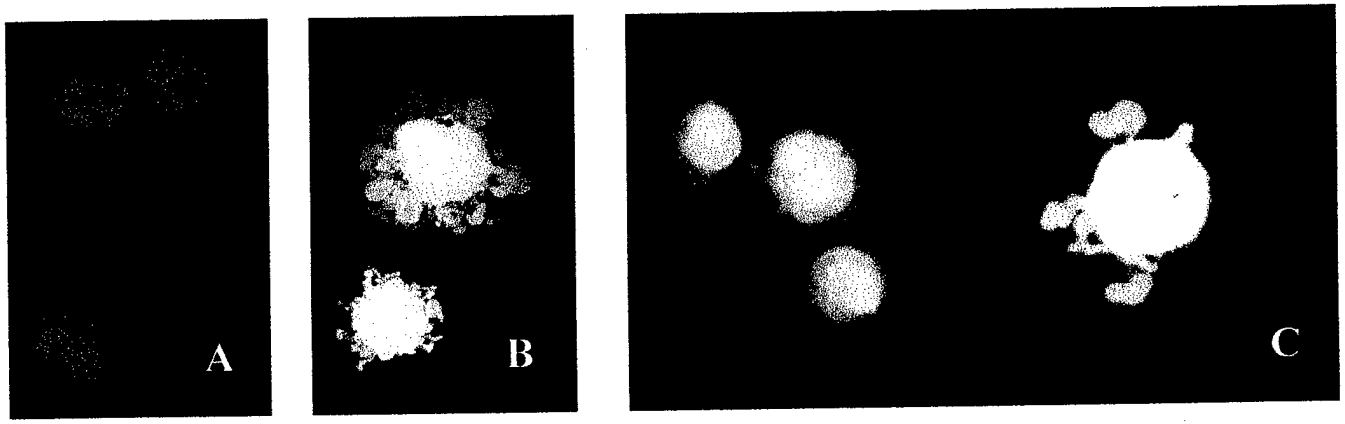


Figure 7

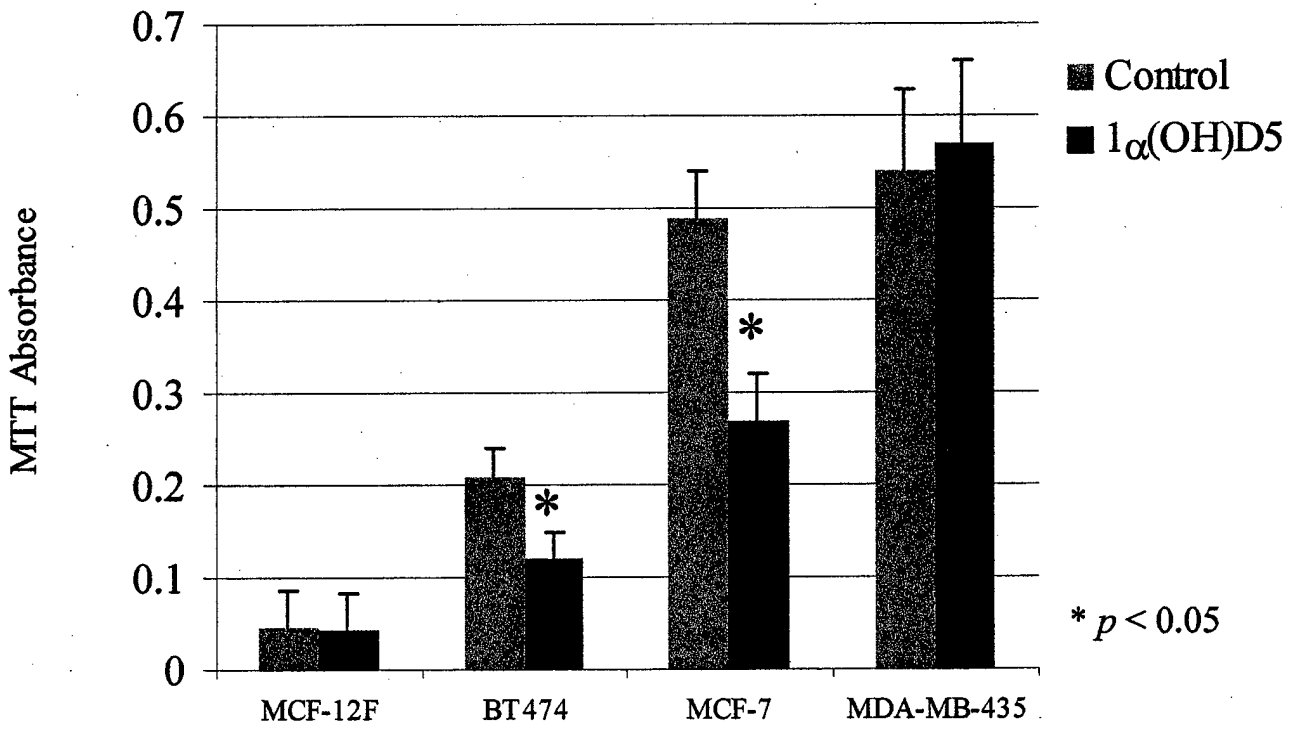

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MTT Absorbance
MCF-12F
BT474
MCF-7
MDA-MB-435
Control
1α(OH)D5
* p < 0.05

Figure 8

Table 1. Effects of 1α(OH)D5 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) †	BW (% gain)
None		6.3	3.6	100
$1,25(OH)_2D3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5 *	8.9	70 *
1α(OH)D5	6.4	6.3	7.2	99
	12.5	6.2	7.2	97
	25.0	6.5	7.1	98
	50.0	ND	ND	113

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

† significance not determined.

Table 2. Chemoprevention of MNU-induced mammary carcinogenesis by 1α(OH)D5 in rats (n = 15).

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

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

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

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

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

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

Table of Contents

Abstract

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* 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 (Friedrich *et al*, 1998; 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 (Friedrich *et al*, 1998) 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 (Hisatake *et al*, 2001; 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 ^1H 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 10 μg/ kg body weight (equivalent to 100 μg 1α(OH)D5/kg

diet for a 150 g rat), 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 binding affinity of 1α(OH)D5 to VDR was determined using competitive binding assays (unpublished data). Results showed that the binding affinity of 1α(OH)D5 in competition with radioactive $1\alpha(OH)_2D_3$ to purified VDR ligand binding domain is 1000-fold less than $1\alpha(OH)_2D_3$ (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 $1\alpha(OH)_2D_3$. 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. We have not yet critically evaluated metabolism and pharmacokinetics of 1α(OH)D5 in target organs.

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. Initiation 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). 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, UISO-BCA-4, 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 to assessment of chemopreventive properties of 1α(OH)D5 in mammary carcinogenesis, we evaluated its efficacy as a possible chemotherapeutic agent. These experiments were carried out in xenograft models as previously described (Mehta *et al*, 2002b). Initial studies were conducted using xenograft of UISO-BCA-4 cells pretreated with 1 μM 1α(OH)D5 for 10 days, which failed to form tumors in athymic (4 weeks old) mice. In other studies, UISO-BCA-4 cells were 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_{DMBA}) 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 affected 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

Results presented in this report on effects of 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

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REFERENCE LIST

Asamoto M, Cohen SM (1994). Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines. Cancer Lett. 83: 201-7.

Atanaskova N, Keshamouni VG, Krueger JS, Schwartz JA, Miller F, Reddy KB (2002). MAP kinase/estrogen receptor cross-talk enhances estrogen-mediated signaling and tumor growth but does not confer tamoxifen resistance. Oncogene. 21: 4000-8.

Berggren M, Gallegos A, Gasdaska JR, Gasdaska PY, Warneke J, Powis G (1996). Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. Anticancer Res. 16: 3459-66.

Bretherton-Watt D, Given-Wilson R, Mansi JL, Thomas V, Carter N, Colston KW (2001). Vitamin D receptor gene polymorphisms are associated with breast cancer risk in a UK Caucasian population. Br J Cancer. 85: 171-5.

Buras RR, Schumaker LM, Davoodi F, Brenner RV, Shabahang M, Nauta RJ, Evans SR (1994). Vitamin D receptors in breast cancer cells. Breast Cancer Res Treat. 31: 191-202.

Carpenter KJ, Zhao L (1999). Forgotten mysteries in the early history of vitamin D. J Nutr. 129: 923-7.

Christakos S (1994). Vitamin D in breast cancer. Adv Exp Med Biol. 364: 115-8.

Coates PJ, Nenutil R, McGregor A, Picksley SM, Crouch DH, Hall PA, Wright EG (2001). Mammalian prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. Exp Cell Res. 265: 262-73.

Demirpence E, Balaguer P, Trousse F, Nicolas JC, Pons M, Gagne D (1994). Antiestrogenic effects of all-trans-retinoic acid and 1α,25-dihydroxyvitamin D_3 in breast cancer cells occur at the estrogen response element level but through different molecular mechanisms. Cancer Res. 54: 1458-64.

Edwards BK, Howe HL, Ries LA, Thun MJ, Rosenberg HM, Yancik R, Wingo PA, Jemal A, Feigal EG (2002). Annual Report to the Nation on the status of cancer, 1973-1999, featuring implications of age and aging on U.S. cancer burden. Cancer. 94: 2766-2792.

Eisman JA, Martin TJ, MacIntyre I (1980). Presence of 1α,25-dihydroxy-vitamin D_3 receptor in normal and abnormal breast tissue. Prog Biochem Pharmacol. 17: 143-50.

Falkenstein E, Norman AW, Wehling M (2000). Mannheim classification of non-genomically initiated rapid steroid actions. J Clin Endocrinol Metab. 85: 2072-5.

Freedman DM, Dosemeci M, McGlynn K (2002). Sunlight and mortality from breast, ovarian, colon, prostate, and non-melanoma skin cancer: a composite death certificate based case-control study. Occup Environ Med. 59: 257-62.

Friedrich M, Rafi L, Tilgen W, Schmidt W, Reichrat J (1998). Expression of 1,25-dihydroxy vitamin D_3 receptor in breast carcinoma. J Histochem Cytochem. 46: 1335-7.

Gallegos A, Gasdaska JR, Taylor CW, Paine-Murrieta GD, Goodman D, Gasdaska PY, Berggren M, Briehl MM, Powis G (1996). Transfection with human thioredoxin increases cell proliferation and a dominant-negative mutant thioredoxin reverses the transformed phenotype of human breast cancer cells. Cancer Res. 56: 5765-70.

Garland FC, Garland CF, Gorham ED, Young JF (1990). Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. Prev Med. 19: 614-22.

Gorham ED, Garland FC, Garland CF (1990). Sunlight and breast cancer incidence in the USSR. Int J Epidemiol. 19: 820-4.

Guyton KZ, Kensler TW, Posner GH (2001). Cancer chemoprevention using natural vitamin D and synthetic analogs. Annu Rev Pharmacol Toxicol. 41: 421-42.

Haussler MR (1986). Vitamin D receptors: nature and function. Annu Rev Nutr. 6: 527-62.

Hisatake J, O'Kelly J, Uskokovic MR, Tomoyasu S, Koeffler HP (2001). Novel vitamin D_3 analog, 21-3-methyl-3-hydroxy-butyl-19-nor D_3, that modulates cell growth, differentiation, apoptosis, cell cycle, and induction of PTEN in leukemic cells. Blood. 97: 2427-33.

Howe HL, Wingo PA, Thun MJ, Ries LA, Rosenberg HM, Feigal EG, Edwards BK (2001). Annual report to the nation on the status of cancer (1973 through 1998), featuring cancers with recent increasing trends. J Natl Cancer Inst. 93: 824-42.

James SY, Williams MA, Kessey SM, Newland AC, Colston KW (1997). The role of vitamin D derivatives and retinoids in the differentiation of human leukemia cells. Biochem Pharmacol. 54: 625-34.

John EM, Schwartz GG, Dreon DM, Koo J (1999). Vitamin D and breast cancer risk: the NHANES I Epidemiologic follow-up study, 1971-1975 to 1992. National Health and Nutrition Examination Survey. Cancer Epidemiol Biomarkers Prev. 8: 399-406.

Jones G, Struguell SA, DeLuca HF (1998). Current understanding of the molecular actions of vitamin D. Physiol Rev. 78: 1193-1231.

Jupe ER, Liu XT, Kiehlbauch JL, McClung JK, Dell'Orco RT (1996). Prohibitin in breast cancer cell lines: loss of antiproliferative activity is linked to 3' untranslated region mutations. Cell Growth Differ. 7: 871-8.

Kelloff GJ, Boone CW, Crowell JA, Steele VE (1996). New agents for cancer chemoprevention. J Cell Biochem. 26 (Suppl): 1-28.

Kirkpatrick DL, Watson S, Kunkel M, Fletcher S, Ulhaq S, Powis G (1999). Parallel syntheses of disulfide inhibitors of the thioredoxin redox system as potential antitumor agents. Anticancer Drug Des. 14: 421-32.

Lazzaro G, Mehta RR, Shilkaitis A, Das-Gupta TK, Mehta RG (1997). Transformation of human breast epithelial cells by DMBA, but not MNU, is accompanied by up-regulation of basic fibroblast growth factor. Oncology Reports. 4: 1175-1180.

Lazzaro G, Agadir A, Qing W, Poria M, Mehta RR, Moriarty RM, Das-Gupta TK, Zhang XK, Mehta RG (2000). Induction of differentiation by 1α(OH)D5 in T-47D breast cancer cells and its interaction with vitamin D receptors. Eur J Cancer. 36: 780-6.

Lundin AC, Soderkvist P, Eriksson B, Bergman-Jungestrom M, Wingren S (1999). Association of breast cancer progression with a vitamin D receptor gene polymorphism. South-East Sweden Breast Cancer Group. Cancer Res. 59: 2332-4.

Mallon E, Osin P, Nasiri N, Blain I, Howard B, Gusterson B (2000). The basic pathology of human breast cancer. J Mammary Gland Biol Neoplasia. 5: 139-63.

Matsutani Y, Yamauchi A, Takahashi R, Ueno M, Yoshikawa K, Honda K, Nakamura H, Kato H, Kodama H, Inamoto T, Yodoi J, Yamaoka Y (2001). Inverse correlation of thioredoxin expression with estrogen receptor- and p53-dependent tumor growth in breast cancer tissues. Clin Cancer Res. 7: 3430-6.

Mehta RR, Bratescu L, Graves JM, Hart GD, Shilkaitis A, Green A, Beattie CW, Das Gupta TK (1992). Human breast carcinoma cell lines: ultrastructural, genotypic, and immunocytochemical characterization. Anticancer Res. 12: 683-92.

Mehta RG, Moriarty RM, Mehta RR, Penmasta R, Lazzaro G, Constantinou A, Guo L (1997a). Prevention of preneoplastic mammary lesion development by a novel vitamin D analog, 1α(OH)D5. J Natl Cancer Inst. 89: 212-8.

Mehta RG, Hawthorne ME, Steele VE (1997b). Induction and prevention of carcinogen-induced precancerous lesions in mouse mammary gland organ culture. Methods Cell Science. 19: 19-24.

Mehta RG, Hawthorne ME, Uselding L, Albinescu D, Moriarty R, Christov K, Mehta RR. (2000a). Prevention of MNU-induced mammary carcinogenesis in rats by 1α(OH)D5. J Natl Cancer Inst. 92: 1836-40.

Mehta RR, Bratescu L, Graves JM, Green A, Mehta RG (2000b). Differentiation of human breast carcinoma cells by a novel vitamin D analog. Int J Oncol. 16: 65-73.

Mehta RG, Mehta RR (2002a). Vitamin D and cancer. J Nutr Biochem. 13: 252-264.

Mehta RG, Hussain EA, Mehta RR, Das-Gupta TK (2002b).

Napoli JL, Fivizzani MA, Schnoes HK, DeLuca HF (1979). Synthesis of vitamin D5: its biological activity relative to vitamin D_3 and D2. Arch Biochem Biophys. 197: 119-25.

Narvaez CJ, Zinser G, Welsh J (2001). Functions of 1alpha,25-dihydroxyvitamin D_3 in mammary gland: from normal development to breast cancer. Steroids. 66: 301-8.

Peto R, Boreham J, Clarke M, Davies C, Beral V (2000). UK and USA breast cancer deaths down 25% in year 2000 at ages 20-69 years. Lancet. 355: 1822.

Pike JW (1991). Vitamin D_3 receptors: structure and function in transcription. Annu Rev Nutr. 11: 189-216.

Polek TC, Weigel NL (2002). Vitamin D and prostate cancer. J Androl. 23: 9-17.

Rachez C, Freedman LP (2000). Mechanism of gene regulation by VDR: a network of coactivator interactions. Genes. 246: 9-21.

Roder JD, Stair E (1999). An overview of cholecalciferol toxicosis. Vet Human Toxicol. 4: 344-348.

Rosenbaum-Smith SM, Osborne MP (2000). Breast cancer chemoprevention. Am J Surg. 180: 249-251.

Stoica A, Saceda M, Fakhro A, Solomon HB, Fenster BD, Martin MB (1999). Regulation of estrogen receptor-alpha gene expression by 1α,25-dihydroxyvitamin D_3 in MCF-7 cells. J Cell Biochem. 75: 640-51.

Storm FK, Mahvi DM, Gilchrist KW (1996). Heat shock protein 27 overexpression in breast cancer lymph node metastasis. Ann Surg Oncol. 3: 570-3.

Swami S, Krishnan AV, Feldman D (2000). 1α,25-dihydroxy-vitamin D_3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells. Clin Cancer Res. 6: 3371-9.

Twentyman PR, Luscombe M (1987). A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer. 56: 279-85.

Vieth R (1999). Vitamin D supplementation, 25(OH)D_3 concentration and safety. Am J Clin Nutr. 69: 842-856.

Vindelov LL, Christensen IJ, Nissen NI (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry. 3: 323-327.

Wang S, Nath N, Fusaro G, Chellappan S (1999). Rb and prohibitin target distinct regions of E2F1 for repression and respond to different upstream signals. Mol Cell Biol. 19: 7447-60.

Welsh J, VanWeelden K, Flanagan L, Byrne I, Nolan E, Narvaez CJ (1998). The role of vitamin D_3 and anti-estrogens in modulating apoptosis of breast cancer cells and tumors. Subcell Biochem. 30: 245-270.

Zajchowski DA, Bartholdi MF, Gong Y, Webster L, Liu HL, Munishkin A, Beauheim C, Harvey S, Ethier SP, Johnson PH (2001). Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res. 61: 5168-78.

Zinser G, Packman K, Welsh J (2002). Vitamin D_3 receptor ablation alters mammary gland morphogenesis. Development. 129: 3067-76.

Figure I. Structure of 1α(OH)D5 and its Ca^{++} mobilizing activity in mammals in relation to other primary vitamin D series compounds.

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

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 [c]	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	100 %	NA
in athymic mice	5	8 ng (s.c.) [b]	6	0 % [a]	NA
UISO-BCA-4 xenograft	5	0.0	6	100 %	NA
in athymic mice	5	12.5 μg/kg diet	6	0 % [a]	NA
BT-474 xenograft in	5	0.0	8.5	0.01 cm^3	NA
athymic mice [d]	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] duration in weeks.
[d] results are expressed as tumor volume(cm^3).

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

Appendix 3: Abstracts presented at various meetings.

EXPERIMENTAL/MOLECULAR THERAPEUTICS 50: Preclinical Toxicology

#5011 **Preclinical Toxicity of 1α-Hydroxyvitamin D5 in Rats and Dogs.** William D. Johnson, Rajeshwari R. Mehta, Robert M. Moriarty, Rajendra G. Mehta, Tapas K. Das Gupta, and David L. McCormick. *IIT Research Institute, Chicago, IL, and University of Illinois, Chicago, IL.*

1α-Hydroxyvitamin D5 [24-ethylcholecalciferol; 1α(OH)D5] is a new vitamin D analog being developed for chemoprevention and therapy of breast cancer. We have previously demonstrated that 1α(OH)D5 induces differentiation and growth inhibition in number of breast cancer cell lines. When administered to rats as a dietary supplement [50μg/kg diet], 1α(OH)D5 reduces the incidence and number of carcinogen-induced mammary carcinomas; chemopreventive activity was achieved without increases in serum calcium or phosphate levels. The present studies were performed to characterize the toxicity of subchronic administration of 1α(OH)D5. CD rats [10/sex/group] and beagle dogs [3/sex/group] received daily oral (gavage) administration of 1α(OH)D5 for four weeks. A complete battery of in-life, clinical pathology, and histopathology evaluations was performed in both studies. In rats, administration of 1α(OH)D5 at doses ranging from 2.5 to 10 μg/kg body weight/day induced no mortality or clinical evidence of toxicity; body weights in treated groups were comparable to those of corn oil controls. Serum calcium demonstrated small, but dose-related and statistically significant increases [11.0 ± 0.46 mg/dL in controls versus 11.6 ± 0.73 mg/dL in the high dose group]; this was not accompanied by renal toxicity and was reversed in recovery animals observed for 2 weeks after cessation of dosing. In dogs, administration of 1α(OH)D5 at 5μ/kg/day induced no clinical evidence of toxicity; doses of 10 μg/kg or above resulted in body weight loss and/or gross toxicity. Dose-related elevations in serum calcium were seen at all dose levels in dogs. These data suggest that the maximum tolerated dose [MTD] for 1α(OH)D5 is > 10 μg/kg/day in rats and 5μg/kg/day in dogs. When considered with published data, these results suggest that the MTD for 1α(OH)D5 in both rats and dogs is higher than is the MTD for its active metabolite, 1α,25 dihydroxyvitamin D3. (Supported by DAMD 17-99-1-9223 and R01-82316)

118 The Inhibition of Nad(P)H:Quinone Oxidoreductase 1 by Mitomycin and ES936 In Mice. Daniel L. Gustafson, Jeffrey C. Rastatter, Jacqueline M. Pal, Patrick J. Kerzic, Michael E. Long, David Siegel, and David Ross. *University of Colorado Health Sciences Center, Denver, CO.*

Previous studies using recombinant proteins and cell lines have shown that mycin C (MMC) can alkylate and inhibit NAD(P)H:quinone oxidoreductase 1 O1) following reductive metabolism by the enzyme. Mechanism-based inac- tion of NQO1 by MMC was pH-dependent, no inactivation of NQO1 was erved at acidic pH (6.0) but inactivation increased as the pH was increased. purpose of the study presented here was to examine time, dose and tissue cific inactivation of NQO1 in mice following MMC treatment. Female balb/C e were treated with a single dose (i.v.) of MMC at either 10 or 20 mg/kg. ues samples (brain, lung, heart, liver, bladder) were removed 1, 2,4 and 24 s post treatment and NQO1 activity was analyzed in cytosolic fractions using marol-inhibitable DCPIP reduction. In separate experiments ES936 (5-me- oxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione), a potent mech- ism-based inhibitor of NQO1, was utilized as a positive control at a dose of 1 /kg (i.p.). NQO1 enzyme activity was significantly inhibited at 1, 2 and 4 hours owing MMC treatment in kidney and lung but NQO1 activity was unchanged in heart, liver and bladder. There was no dose dependency in the observed bition at the doses used in this study and NQO1 activity was restored by 24 rs. Studies using ES936 demonstrated significant inhibition of NQO1 activity tissues except bladder. ES936 treatment resulted in greater than 90% bition in some tissues, and at least 50% in all tissues analyzed. MMC treat- resulted in a maximum of 40% inhibition in any tissue at any time. These lts demonstrate that MMC can serve as a substrate for NQO1 in vivo and abolism of MMC by NQO1 resulting in enzyme inactivation may be tissue cific (supported by CA75955, CA51210).

119 Effects of 1α-Hydroxyvitamin D5 Are Selective to Malignant ast Epithelial Cells. Rajeshwari R. Mehta, Konstantin Christov, and Rajendra Mehta. *University of Illinois, Chicago, IL.*

ecently we showed that a relatively non-toxic analog of vitamin D, 1α-hy- oxyvitamin D5 (1α(OH)D5) has potent cell differentiating and growth inhibitory ons in various human breast carcinoma cell lines. In this study, we compared ffects of 1α(OH)D5 on malignant and nonmalignant human breast tumors nonmalignant breast tissues. Breast tissues were obtained from women ergoing mastectomy or lumpectomy procedures. Effect of 1αOH)D5 was mined in in vitro explant culture. Fresh tissues, received from the operating m were minced in to small pieces and incubated for 0-72 hours at 37C in ure medium containing charcoal stripped FBS alone or medium containing h 1α(OH)D5. Normal human breast tissues incubated in the basal medium 1α(OH)D5 were viable for 72 hours. Occasionally presence of cells with notic nuclei was observed in both control and treated tissues. Originally at 0,4-6 cells /alveolar structure showed Ki-67 staining indicating active pro- ation of epithelial cells. VDR immunostaining was observed in 60-70% of cells. change in Ki-67, or VDR was observed at 72 hours in control and 1α(OH)D5 ted tissues. Fibroadenoma tissue samples maintained in culture for 72 hours served original essential architecture of the alveolar, ductal and stromal com- ents. However, when the 1α(OH)D5 was added to the culture, a proportion of alveolar structures lost their epithelial components. The individual cell showed olysis and nuclear pyknosis in some epithelial components. Ki-67 immuno- ctivity was similar in control and treated tissues however, VDR staining was eased in treated tissues (90%) as compared to control (30-40%) . Human ast tumors incubated in the basal medium preserved the original histopatho- ical features up to 48 hours. Following incubation with 1α(OH)D5, increased optosis and cell death was observed. Immunoreactivity to Ki-67 was de- ased significantly in vitamin treated tumors. We also observed increased VDR ression in 1α(OH)D5 treated cells. These results suggest that human breast ues maintain original tissue architecture for up to 48 hours in vitro and 2) OH)D5 has no effect on normal breast tissue but induces apoptosis in and ignant breast tumors. (Supported by DOD17-99-1-9223 and NCI R01-82316)

120 1α-Hydroxy Vitamin D5 Induces Apoptosis and Cell Cycle Arrest in 474 Breast Cancer Cells. Erum A. Hussain, Krishna P. Bhat, Rajeshwari R. ta, and Rajendra G. Mehta. *University of Illinois at Chicago, Chicago, IL.*

reast cancer is the second major cause of cancer deaths among women in the Several epidemiological studies have shown an increased risk of breast er mortality with lower serum vitamin D levels. It is well established that the ve metabolite of vitamin D, 1α,25$(OH)_2D_3$ (VD_3), induces cell differentiation inhibits cellular proliferation *in vitro* at concentrations that would be hyper- emic *in vivo*. Therefore, various non-calcemic analogs of VD_3 have been thesized and characterized. Previously, we had reported efficacy of a vitamin alog, 1α-hydroxy-24-ethyl cholecalciferol (VD_5), in inhibiting growth of estro- receptor positive (ER^+) breast cancer cells and preventing development of BA-induced preneoplastic lesions in mouse mammary organ culture. In addi- , VD_5 reduces tumor incidence and multiplicity in carcinogen-treated rats. To erstand the mechanism of action of VD_5, we used steroid hormone receptor itive BT-474, and steroid hormone non-responsive MDA-MB-231 and MDA- 435 breast cancer cell lines as well as non-tumorigenic MCF-12F cell line. treatment resulted in significant cell growth inhibition in BT-474 cells (58.9 % ontrol). No significant growth inhibition was seen in MCF-12F, MDA-MB-435, or MDA-MB-231 cells. Acridine Orange/Ethidium Bromide staining showed marked apoptosis in BT-474 cells with VD_5 treatment while MDA-MB-435 and MCF12F showed no difference from control. Cell cycle analysis confirmed the growth inhibition induced by VD_5 in BT-474 cells; G-1 phase was 141.2% of control while S phase was reduced by 66.2%. Immunohistochemical analysis showed down-regulation of progesterone receptor with VD_3 and VD_5 treatment in BT474 cells with no apparent effect on the expression of vitamin D receptor (VDR) or ER. BT-474 cells have high expression of VDR, which suggests that growth inhibitory effects of VD_5 may be mediated through VDR or VD_5-directed down-regulation of progesterone receptors. Further studies on the mechanism of action of VD_5 are warranted to explore its potential use in breast cancer treatment. (Supported by NCI CA-82316 and DAMD-17-99-1-9223).

#1121 Anti-Proliferative Effects of 1α,25-Dihydroxyvitamin D3 and Vitamin D Analogs on Murine Endothelial Cells. Ronald J. Bernardi, Ruth A. Modzelewski, Donald L. Trump, and Candace S. Johnson. *University of Pittsburgh Cancer Institute, Pittsburgh, PA.*

Our laboratory and others have shown previously that the active form of vitamin D, calcitriol (1,25-D_3), is a potent anti-tumor agent in a variety of model systems *in vitro* and *in vivo*. While there is strong evidence that 1,25-D_3 inhibits the growth of tumor cells directly, inhibition of angiogenesis may also play an important role in mediating the anti-tumor effects of 1,25-D_3 *in vivo*. To test this hypothesis, we assessed the ability of 1,25-D_3 and the analogs EB1089, Ro-25-6760, and Ilex-7553 to inhibit the growth of endothelial cells in culture. We also used an angiogenesis specific microarray to assess the effects of 1,25-D_3 on angiogenic signaling between tumor and endothelial cells. First, murine endothelial cells from a variety of tumor types and normal tissues were isolated by fluorescence-activated cell sorting for endothelial cell markers. Then, we used a crystal violet assay to assess the growth inhibitory effects of 1,25-D_3 and its analogs. In addition, we performed western blots on whole cell lysates to assess vitamin D receptor (VDR) protein levels, as well as markers of cell cycle. We demonstrate that 1,25-D_3 inhibits the growth of tumor-derived endothelial cells (TDECs) from two different tumor models at nanomolar concentrations, but has little effect on aortic or yolk sac endothelial cells. EB1089 and Ro-25-6760 were similar to 1,25-D_3 in their potency, while Ilex-7553 was significantly more potent. VDR and $p27^{Kip1}$ protein levels were increased in the TDECs by 1,25-D_3, but not in the aortic endothelial cells. Preliminary results from the microarray experiments have identified putative targets that are modulated by 1,25-D_3 in tumor cells, while few changes were seen in the TDECs. In conclusion, our results demonstrate that 1,25-D_3 and its analogs can directly inhibit the proliferation of tumor-derived endothelial cells at concentrations comparable to those that are required to inhibit tumor cells. Furthermore, angiogenic signaling from tumor cells to endothelial cells may also be affected by 1,25-D_3. Thus, our work supports the hypothesis that angiogenesis inhibition plays a significant role in the anti-tumor effects of 1,25-D_3 and its analogs *in vivo*. This work has been supported by NIH grant CA67267, CaPCURE, The M.H. Jennings Fnd., and USAMRMC 17-98-1-8549.

#1122 Antiproliferative Activity of G-Quartet Forming Oligonucleotides with Backbone and Sugar Modifications. Virna Dapic, Paula J. Bates, John O. Trent, Shelia D. Thomas, and Donald M. Miller. *University of Louisville, Louisville, KY.*

GRO29A is a G-rich 29-mer, stabilized by G-quartet formation. It has dramatic antiproliferative activity in prostate cancer cells in vitro. We have shown that the effect of this oligonucleotide corresponds to binding to a 106kDa protein, which is most likely nucleolin. Here we examine antiproliferative activity, protein binding, and G-quartet formation of G-rich oligonucleotides, analogs of GRO29A. These include analogs with phosphorothioate (PS29A), 2-'O-Methyl RNA (MR29A), and mixed DNA/2'O-Methyl RNA (MRdG29A). We have shown by UV spectroscopy that all of the modified analogs form stable structures, consistent with G-quartet formation. Oligonucleotides PS29A and MRdG29A were able to significantly inhibit proliferation in DU145 prostate cancer cell line (70-80%), whereas MR29A had a much weaker effect (20%). These results were reproducible in MDA-MB231 breast cancer cells and in HeLa, cervical cancer cells. As was the case with GRO29A, the growth inhibitory oligonucleotides were able to compete with a telomere sequence oligonucleotide for binding to a specific cellular protein, which we presume to be nucleolin. The less active MR29A did not compete significantly for this protein. In addition, we have investigated the stability of these G-rich oligonucleotides in the presence of serum-containing medium. We find that all GRO29A analogs, including an unmodified DNA phosphodiester oligonucleotide are extremely stable under these conditions, indicating that secondary structure plays an important role in biological stability. We believe that nucleolin binding to these oligonucleotides results in inhibition of at least one of the multiple functions of this interesting protein. These data suggest a strong potential for further development of these G-rich oligonucleotides as therapeutic agents.

#1123 Specific Inhibition of C-Myc Expression by the Cationic Porphyrin TMPyP4 Results in Downregulation of hTERT Expression and Reduced Telomerase Activity. Cory Lyle Grand, David J. Bearss, Haiyong Han, Ruben Munoz, Daniel D. von Hoff, and Laurence H. Hurley. *University of Arizona, Tucson, AZ.*

Telomerase activity is associated with escape from cellular senescence and can confer the immortality implicated in the process of malignant transformation