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14. ABSTRACT The two most significant achievements in this reporting period are : launching of studies to evaluate the molecular mechanism/s of action of . 1,25-dihydroxyvitamin D3-3-bromoacetate (1,25(OH)2D3-3-BE), and development of an androgen-sensitive mouse model of human prostate cancer. In addition we have screened several cancer cell lines to determine potential efficacy of 1,25(OH)2D3-3-BE in cancers, in addition to prostate cancer.					
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Introduction

Therapeutic potential of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in prostate cancer is well-recognized. However, its clinical use has been restricted by its inherent calcemic toxicity. In recent studies we demonstrated that 1 α ,25-dihydroxyvitamin D₃-3-bromoacetate [1,25(OH)₂D₃-3-BE], a derivative of 1,25(OH)₂D₃ that covalently links 1,25(OH)₂D₃ inside the ligand-binding pocket of nuclear vitamin D receptor (VDR) is a strong antiproliferative and pro-apoptotic agent in several androgen-sensitive and androgen-refractory human prostate cancer cells. Furthermore, 1,25(OH)₂D₃-3-BE demonstrated strong anti-prostate tumor effect in athymic mice without toxicity. The goal of this project is to evaluate the translational potential of 1,25(OH)₂D₃-3-BE as a therapeutic agent for prostate cancer. This will be achieved by determining the efficacy of 1,25(OH)₂D₃-3-BE in mouse models of human androgen-sensitive and androgen-insensitive prostate cancer, as well as evaluating its molecular mechanisms of action in several *in vitro* studies.

Studies completed/goals achieved during the one year period (Year 2) of the project

Studies to evaluate mechanism of action of 1,25(OH)₂D₃-3-BE in prostate cancer cell lines

1. Modulation of 1,25-dihydroxyvitamin D₃-24-hydroxylase (24-OHase) gene by 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE in LNCaP androgen-sensitive human prostate cancer cells

1,25(OH)₂D₃-3-BE is an alkylating agent and thus, in addition to alkylating VDR, it can potentially alkylate various cellular proteins non-specifically. The demonstration that cellular activities of 1,25(OH)₂D₃-3-BE are mediated by VDR is an essential facet of our studies to evaluate the mechanisms of the cellular properties of this compound. Several genes, including osteocalcin, osteopontin and 1,25-dihydroxyvitamin D₃-24-hydroxylase (CYP24) are known to be induced by 1,25(OH)₂D₃. In an earlier study we demonstrated that 1,25(OH)₂D₃-3-BE induces mRNA for osteocalcin and CYP24 in human keratinocytes. In the current study we evaluated whether 1,25(OH)₂D₃-3-BE is capable of modulating the expression of CYP24, similar to 1,25(OH)₂D₃ in LNCaP androgen-sensitive human prostate cancer cells.

The CYP24 gene product catalyzes the introduction of a hydroxyl group at the 24-position in 1,25(OH)₂D₃, followed by multiple oxidations of the side chain leading to calcitroic acid, the final catabolite that is excreted. Therefore, CYP24 is the initiator of the catabolic degradation of 1,25(OH)₂D₃. We hypothesize that covalent attachment of 1,25(OH)₂D₃-3-BE into the ligand binding pocket of VDR will decrease its catabolism. In essence it would require more 1,25(OH)₂D₃-3-BE to induce the same level of CYP24 message as 1,25(OH)₂D₃.

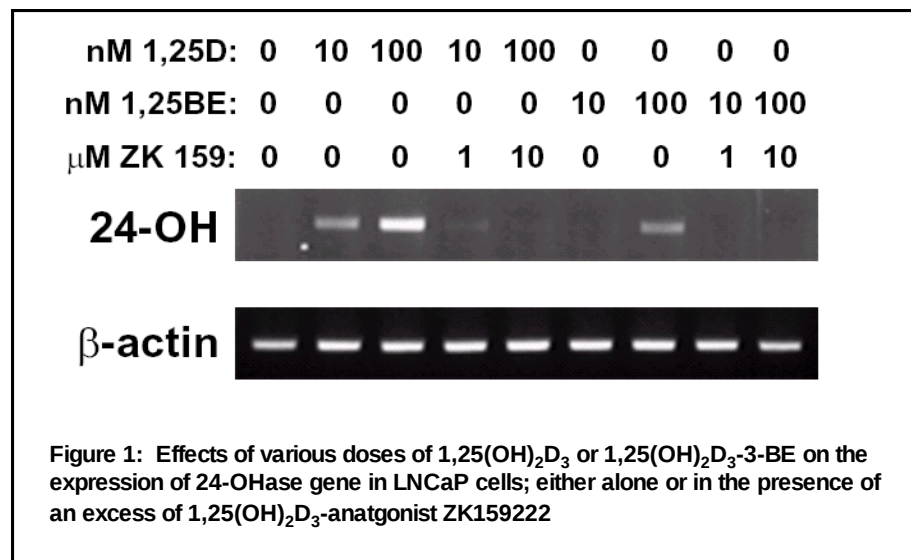
To further investigate the requirement for VDR in 1,25(OH)₂D₃-3-BE action, we used a specific VDR antagonist, ZK159222 which has been shown to be effective in blocking VDR-mediated gene regulation. Therefore, LNCaP cells were treated with either 1,25(OH)₂D₃-3-BE or ZK 15922 alone or in combination, and the levels of CYP24 mRNA analyzed by RT-PCR.

Experimental procedure: LNCaP cells were plated at 3 x 10⁵ cells per well of a six well dish. The following day, the cells were treated with various concentrations of 1,25(OH)₂D₃ or 1,25(OH)₂D₃-3-BE. Sixteen (16) hours later, total RNA was prepared by Trizol extraction (Invitrogen, Carlsbad, CA). One microgram of total RNA was subjected to reverse transcription

using moloney murine leukemia virus (MMLV) reverse transcriptase under standard conditions. Following cDNA synthesis, 1/10th of the reaction was subjected to PCR using gene specific primers to both 24-hydroxylase and beta-actin. The products were analyzed on a 1% agarose gel. In a separate experiment LNCaP cells were treated with various doses of 1,25(OH)₂D₃ or 1,25(OH)₂D₃-3-BE, either alone or in the presence of various concentrations of ZK 159222.

Results: The results of this experiment, shown in **Figure 1**, demonstrate that treatment of LNCaP cells with 10⁻⁷M of 1,25(OH)₂D₃ strongly induces CYP24-message, and moderately with 10⁻⁸M. In the case of 1,25(OH)₂D₃-3-BE, induction of CYP24 mRNA is observed only at 10⁻⁷M, and intensity of message is similar to 10⁻⁸M of 1,25(OH)₂D₃. These results demonstrate a

log scale difference in inducing the message for CYP24 between 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE.



Furthermore, with ZK15922, CYP24-message (by 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE) was reduced in a dose-dependent fashion, and completely obliterated with 10⁻⁴M (of ZK15922).

In an earlier study we demonstrated that ¹⁴C-1,25(OH)₂D₃-3-BE specifically labeled VDR in extracts of ROS 17/2.8 bone cells and calf thymus nuclear cytosol, indicating that labeling by 1,25(OH)₂D₃-3-BE is VDR-specific. Therefore, collectively these results strongly suggest that **cellular effects of 1,25(OH)₂D₃-3-BE in LNCaP prostate cancer cells are VDR-dependent.**

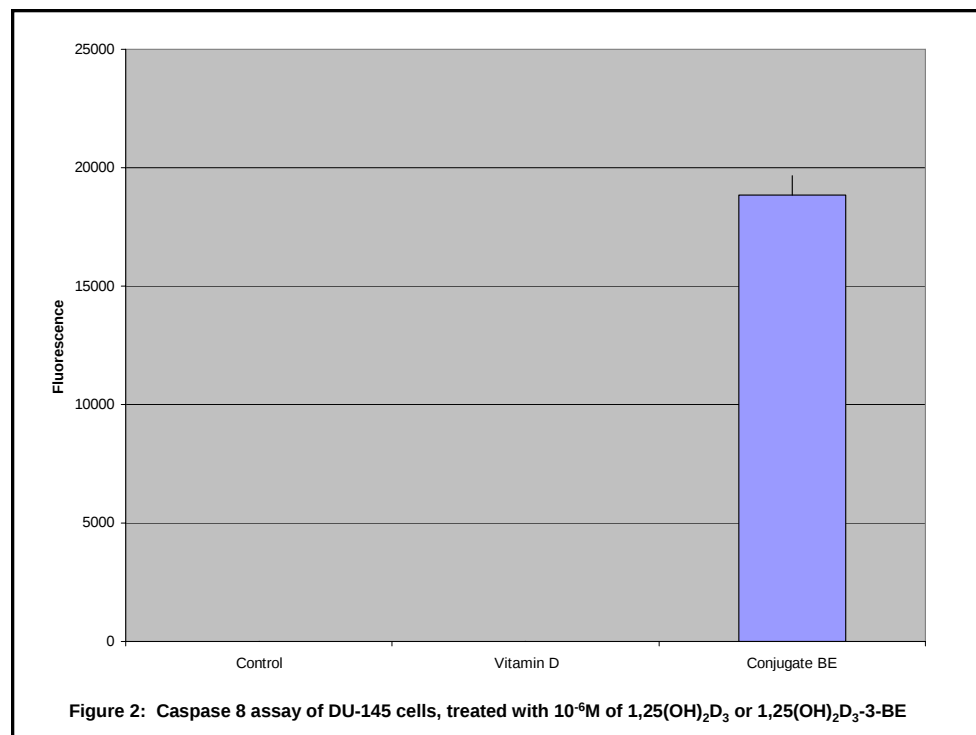
Mechanism of apoptosis by 1,25(OH)₂D₃-3-BE:

(i) Modulation of caspase 8 in DU 145 cells

Earlier we have demonstrated that 1,25(OH)₂D₃-3-BE induces apoptosis (programmed cell-death) in prostate cancer cells. Caspase 3, 8 and 9 (and other caspases) are key indicators of apoptosis in cells. For example, caspase 3 is activated during the cascade of events during apoptosis. It cleaves a variety of molecules containing DEVD amino acid motif. Such molecules include poly-ADP-ribose polymerase (PARP), U1-ribonucleoprotein etc. Caspase 8 is an upstream caspase, and its activation leads to the activation of additional caspases and subsequent cleavage of PARP and other molecules. Caspase 9 is a key regulator of apoptosis *in vivo*. Activation of caspase 9 activates pro-caspase 3, which in turn is manifested through classical features of apoptosis such as cleavages of PARP, U1-ribonucleoprotein etc.

Experimental procedure: DU145 cells were grown in medium to approximately 60% confluence when they were treated for 6 hours with 10⁻⁶M of either 1,25(OH)₂D₃ or 1,25(OH)₂D₃-3-BE. A control plate was run with ethanol-control. After this treatment Caspase-

8 assay was performed using Caspase colorimetric assay kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. Briefly, cells were collected by centrifugation at 1000 RPM for 5min. The cell pellet was lysed with lysis buffer, and the lysate was incubated



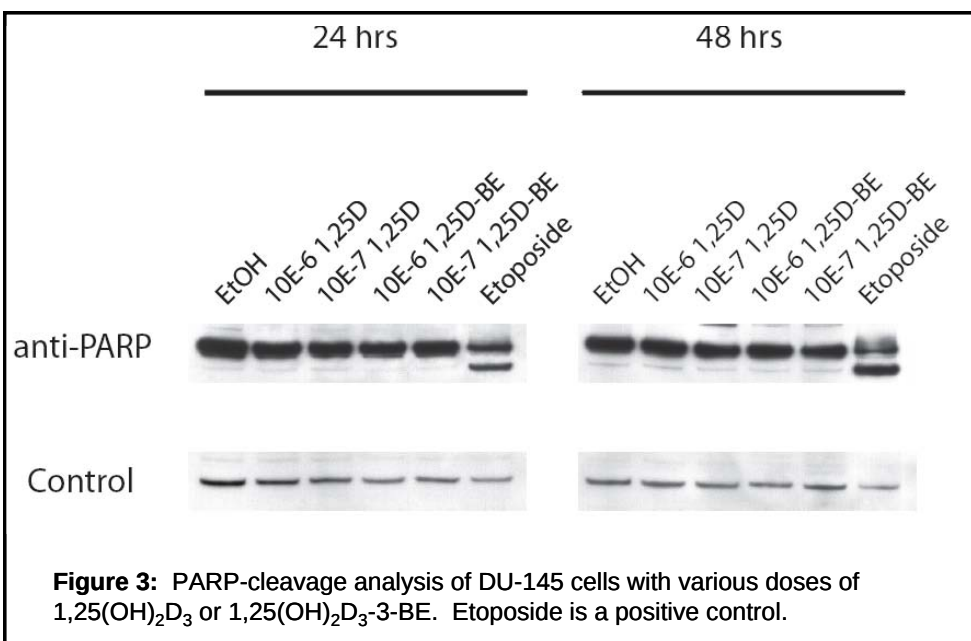
on ice for 10 min. and centrifuged for 10,000 RPM for 5 min. Protein was estimated using Bradford protein estimation kit (BioRad). The enzymatic reactions were carried out in a 96 well plate. For each reaction 100 μg lysate protein in 50 μl was incubated with 50 μl of 2 X reaction buffer and 5 μl of caspase-8 colorimetric

substrate for 2h at 37°C . The absorbance was determined at 405 nm.

Results: As shown in Figure 2, $1,25(\text{OH})_2\text{D}_3$ -3-BE caused a strong elevation of caspase 8, while an equivalent amount of $1,25(\text{OH})_2\text{D}_3$ failed to do so (similar to ethanol-control). This results suggests that apoptotic activity of $1,25(\text{OH})_2\text{D}_3$ -3-BE is mediated through activation of caspase-pathway.

Evaluation of PARP-cleavage by $1,25(\text{OH})_2\text{D}_3$ -3-BE in DU-145 cells

We specified earlier that caspase-activation leads to apoptosis which is manifested via



cleavage of poly-ADP-ribose polymerase (PARP), U1-ribonucleoprotein etc. Since $1,25(\text{OH})_2\text{D}_3$ -3-BE activated caspase-8 (Figure 2), we carried out a Western Blot analysis of DU-145 cells treated with various doses of either $1,25(\text{OH})_2\text{D}_3$ or $1,25(\text{OH})_2\text{D}_3$ -3-BE, followed by

Western Blot analysis with PARP-antibody. As shown in Figure 3, neither 1,25(OH)₂D₃-3-BE nor 1,25(OH)₂D₃ induced PARP-cleavage, while etoposide (positive control) clearly demonstrated cleavage of the PARP protein. These results indicate that apoptosis in DU-145 cells, induced by 1,25(OH)₂D₃-3-BE may involve a PARP-independent pathway.

Development of a mouse xenograft model of androgen-sensitive human prostate tumor

Our overall goal for this project is to determine the efficacy of 1,25(OH)₂D₃-3-BE in both androgen-sensitive, as well as androgen –insensitive human prostate tumor. In the previous year we have developed a mouse xenograft model for androgen-insensitive (DU-145) prostate tumor. During the reporting year we have developed the same for androgen-sensitive LNCaP prostate cancer cells. The procedure is given below.

Procedure: Six (6) weeks old male athymic mice, weighing approximately 20 gm (Charles River) were kept on normal animal diet and water *ad libitum*. LNCaP human androgen-sensitive prostate cancer cells were grown in DMEM media containing 5% FBS. When the cells grew to confluence, they were trypsinized and centrifuged. The cell pellet was suspended in PBS. An aliquot was counted for cell-number. Approximately 5 x 10⁶ cells/ 100 µl of suspension was mixed with an equal volume Matrigel (a basement matrix, required for tumor-development) and 200 µl of the mixture was injected (with a 26 gauge needle) under the skin in the flank of the mice. Palpable tumor with an approximate size of 1 mm³ was obtained within two weeks post-injection.

Screening of cancer cells, other than prostate cancer for anti-proliferative activity with 1,25(OH)₂D₃-3-BE

Similar to what we did in the first year (of this project), we continue to screen cancer cells, other than prostate cancer to determine efficacy (or lack thereof) of 1,25(OH)₂D₃-3-BE in various cancers. As a general procedure we treated culture-grown cells with various doses of either 1,25(OH)₂D₃-3-BE or 1,25(OH)₂D₃ followed by ³H-thymidine incorporation assay

(procedure given below).

³H-thymidine-incorporation assay:

After the treatment media was removed from the wells and replaced with media containing ³H-thymidine (0.1µCi) per well, and the cells were incubated for

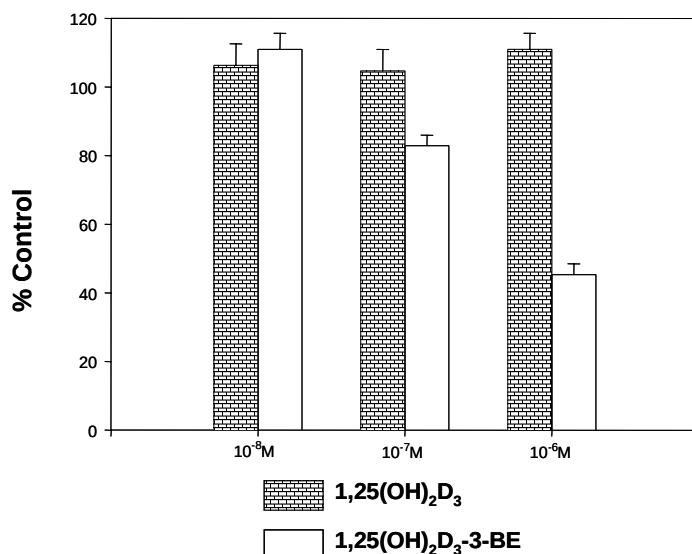
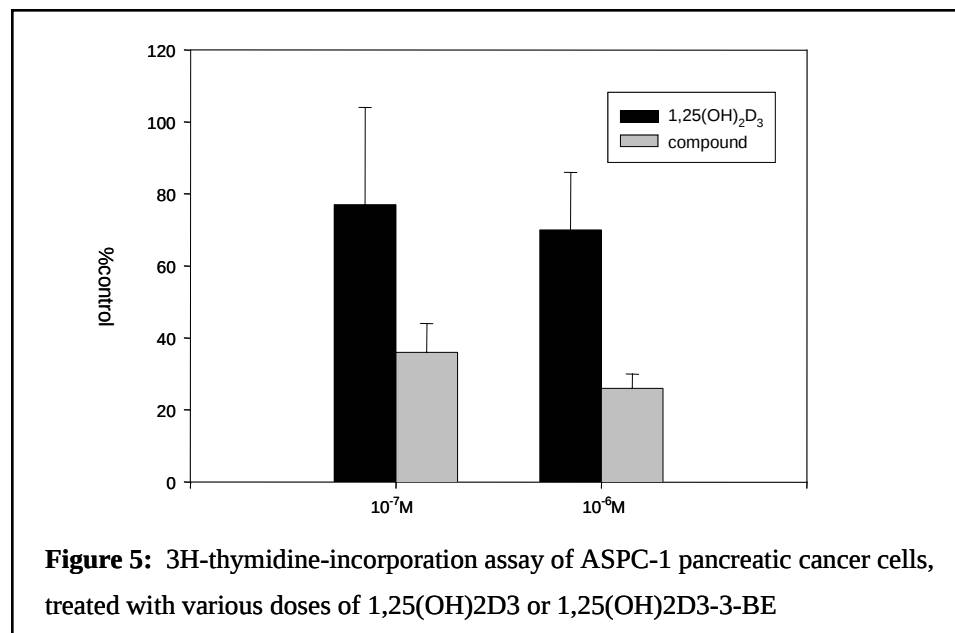


Figure 4: ³H-thymidine-incorporation assay of HS-766 pancreatic cancer cells, treated with various doses of 1,25(OH)₂D₃ or 1,25(OH)₂D₃-3-BE

3 hours at 37⁰C. After this period media was removed by aspiration and the cells were washed

thoroughly (3 X 0.5 ml) with PBS. Then ice-cold 5% perchloric acid solution (0.5 ml) was



added to each well and the cells were incubated on ice for 20 minutes. After this incubation, perchloric acid was removed by aspiration, replaced with 0.5 ml of fresh perchloric acid solution and the cells were incubated at 70°C for 20 minutes. Solution from each well was mixed with scintillation fluid and counted in a liquid scintillation

counter. There were eight (8) wells per sample, and statistics were carried out by Student's t test. Results are expressed as percent incorporation of radioactivity versus ethanol-control.

Results

As shown in Figures 4 and 5, 1,25(OH)₂D₃-3-BE has strong antiproliferative effect in two pancreatic cancer cell-lines (in addition to several kidney cancer cell-lines, delineated in the Year 1 report), demonstrating its potential as a therapeutic agent for kidney and pancreatic cancers, in addition to prostate cancer.

Plans for the immediate future: Currently we are in the process of developing a study to determine maximum tolerated dose (MTD) of 1,25(OH)₂D₃-3-BE in a mouse model. Once that is established we will order a large number of athymic mice for our proposed efficacy study of 1,25(OH)₂D₃-3-BE in both androgen-sensitive and androgen-insensitive mouse xenograft models.

During the past one year our efforts with 1,25(OH)₂D₃-3-BE and a related has generated two (2) abstracts and one publication that are included in the appendix.

KEY RESEARCH ACCOMPLISHMENTS

- Launched several mechanistic studies to evaluate the molecular pathway of action of 1,25(OH)₂D₃-3-BE.
- Developed a mouse xenograft model for androgen-sensitive prostate tumor.
- Determined antiproliferative activity of 1,25(OH)₂D₃-3-BE in pancreatic cancer cell line to evaluate potential therapeutic option for this compound.

REPORTABLE OUTCOME

During the past one year our efforts with 1,25(OH)₂D₃-3-BE and a related has generated one publication, three (3) abstracts and an invited lecture (included in the appendix).

CONCLUSION

Our effort for the past one year has established the groundwork for the current year and beyond to develop 1,25(OH)₂D₃-3-BE and related compounds for prostate and other cancers.

APPENDIX

Meeting Abstract 1:

IMPACT meeting, Atlanta, GA, September 5-8, 2007

A NOVEL VITAMIN D COMPOUND FOR PROSTATE CANCER

Rahul Ray; James Lambert (University of Colorado Health Science Center, Aurora, CO), Sibaji Sarkar, Kelly S. Persons.

Numerous epidemiological studies have demonstrated the importance of dietary vitamin D in preventing various cancers, including prostate cancer. In addition, therapeutic potential of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the biologically active metabolite of vitamin D, and its analogs in cancer is well-documented. However, inherent calcemic toxicity of this hormone, particularly at therapeutic doses, has prevented its general use as an anticancer agent, and opening the door for the development of vitamin D analogs with potent antiproliferative activity and reduced systemic toxicity.

Prostate cancer cells respond to 1,25(OH)₂D₃ by decreasing proliferation and enhancing differentiation. These cell-regulatory processes result from a strong and specific interaction between 1,25(OH)₂D₃ and vitamin D receptor (VDR) present in the nucleus of the tumor cells. With financial support from the Department of Defense Prostate Cancer Research Program, Fiscal Year 2005 Idea Development Award we have developed a novel derivative of 1,25(OH)₂D₃ [1,25-dihydroxyvitamin D₃-3-bromoacetate, 1,25(OH)₂D₃-3-BE] that covalently attaches 1,25(OH)₂D₃ inside the ligand-binding pocket of VDR. We hypothesized that covalent attachment of 1,25(OH)₂D₃ (via 1,25(OH)₂D₃-3-BE) inside the VDR-binding pocket will not allow the catabolic enzymes to degrade 1,25(OH)₂D₃, and effectively increase the potency of 1,25(OH)₂D₃. As a result lesser amount of 1,25(OH)₂D₃-3-BE will be required for tumor-reduction with diminished toxicity.

In vitro assays of 1,25(OH)₂D₃-3-BE demonstrated that this compound has strong growth-inhibitory property in several hormone-sensitive and hormone-insensitive prostate cancer cells (LNCaP, PC-3, DU-145, LAPC-4). This growth-inhibitory effect is considerably stronger than an equimolar amount of 1,25(OH)₂D₃. Furthermore, we observed that 1,25(OH)₂D₃-3-BE induces programmed cell death or apoptosis in these cells (contrary to 1,25(OH)₂D₃) as demonstrated by fragmentation of nuclear DNA and activation of pro-apoptotic caspases. Most importantly preliminary *in vivo* studies showed that 1,25(OH)₂D₃-3-BE strongly reduces androgen-refractory prostate tumor (DU-145) in a mouse xenograft model without causing significant toxicity. These results demonstrate strong therapeutic potential of 1,25(OH)₂D₃-3-BE in prostate cancer.

Aphios Corporation, Woburn, MA has procured the exclusive right to use this compound in prostate cancer. They are in the process of making liposomal preparations of 1,25(OH)₂D₃-3-BE in anticipation of clinical trial. Furthermore, 1,25(OH)₂D₃-3-BE has shown very strong promise in pancreatic, renal and bladder cancers.

In summary, we have used funds from DOD, PCRFP to develop a vitamin D compound with strong therapeutic and commercial potential for prostate and other cancers.

IMPACT: No therapy is currently available for prostate cancer, localized or metastasized that fail to respond to androgen therapy. Our results demonstrate that 1,25(OH)₂D₃-3-BE, a derivative of 1,25(OH)₂D₃, has a strong therapeutic potential in such malignancies.

Meeting Abstract 2:

Nano Science and Technology Institute, Annual meeting, Santa Clara, CA, May 20-24, 2007.

Nanosomal formulation of a vitamin D receptor alkylating compound for prostate cancer.

Rahul Ray¹, and Trevor Castor². ¹Boston University School of Medicine, Boston, MA 02118, 617-638-8199, FAX 617-638-8194, bapi@bu.edu, and ²Aphios Corporation, Woburn, MA 01801, 781-932-6933, FAX 781-932-6865, tcaster@aphios.com (**Cancer Ligands**)

Prostate cancer is the second leading cause of cancer death in men in the US. The mainstay of chemotherapy includes androgen-deprivation. However, no therapy is currently available for prostate cancer, localized or metastasized that fail to respond to androgen therapy. In addition to androgens, prostate cancer cells respond to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) by decreasing proliferation and enhancing differentiation. Furthermore, most cancer cells contain nuclear vitamin D receptor (VDR) that is responsible for the biological actions of 1,25(OH)₂D₃. However, use of 1,25(OH)₂D₃ has been seriously limited by risk of hypercalcemia and hypercalciuria at pharmacological doses.

Interaction between 1,25(OH)₂D₃ and VDR is an equilibrium process. Therefore, in the steady state a finite amount of free 1,25(OH)₂D₃ (not bound to VDR) is always present in the equilibrium mixture, which undergoes rapid catabolic degradation. From a therapeutic standpoint such catabolic degradation is met clinically with high doses that cause toxicity. Therefore, if catabolic degradation of 1,25(OH)₂D₃ is reduced or eliminated its therapeutic potency can be enhanced significantly.

1 α ,25-Dihydroxy vitamin D₃-3-bromoacetate (1,25(OH)₂D₃-3-BE) is a derivative of 1,25(OH)₂D₃ which reacts specifically with a single Cysteine residue in the ligand binding pocket of VDR. We argued that, 1,25(OH)₂D₃-3-BE, once covalently linked to VDR, cannot exit the binding pocket; making it an irreversible process. Furthermore, 1,25(OH)₂D₃-3-BE, covalently linked inside VDR binding pocket is prevented from interacting with catabolic enzymes. We hypothesized that such a process will increase the effective concentration of 1,25(OH)₂D₃; and lesser amount of 1,25(OH)₂D₃-3-BE will be required for tumor-reduction with diminished toxicity.

In vitro tests of 1,25(OH)₂D₃-3-BE demonstrated that this compound has strong growth-inhibitory and apoptosis-inducing properties in several hormone-sensitive and hormone-insensitive prostate cancer cells (LNCaP, PC-3, DU-145). This growth-inhibitory effect was significantly stronger than an equimolar amount of 1,25(OH)₂D₃. Furthermore, 1,25(OH)₂D₃-3-BE induces apoptosis in these cells contrary to 1,25(OH)₂D₃. Additionally, preliminary *in vivo* studies showed that 1,25(OH)₂D₃-3-BE is of low-toxicity, and it strongly reduces androgen-refractory prostate tumor (DU-145) in a mouse xenograft model. On a molar basis 1,25(OH)₂D₃-3-BE is approximately six (6) times stronger than 1,25(OH)₂D₃ in reducing tumor-size. It is also significantly less toxic than an equimolar amount of 1,25(OH)₂D₃. Therefore, 1,25(OH)₂D₃-3-BE has a strong therapeutic potential in prostate cancer.

Phospholipid nanosomes are small, uniform liposomes that are made up of biocompatible phospholipids and cholesterol; and they are designed to encapsulate vitamin D drugs in the lipid-bilayer of the nanosomes. Nanosomal formulation of 1,25(OH)₂D₃-3-BE is designed to further reduce systemic toxicity, increase circulatory half-life and efficacy resulting in an improved therapeutic index for this vitamin D-based drug in prostate cancer.

In summary, novelty of our approach is development of a derivative of 1,25(OH)₂D₃ (1,25(OH)₂D₃-3-BE) to kinetically engage VDR to increase the half-life of 1,25(OH)₂D₃, and increase its potency. Nanosomal formulation will further increase its circulatory half-life and efficacy, and decrease toxicity.

Meeting Abstract 3:

Fourteenth Brown University Symposium on vitamin D, Providence, RI, June 22-23, 2007

Abstract:

Harnessing the pharmacodynamic properties of vitamin D analogs: an old wine in a new bottle. Rahul Ray, Ph.D., Boston University School of Medicine, Boston, MA

Therapeutic potential of $1,25(\text{OH})_2\text{D}_3$ -analogs depends on their pharmacodynamic/ pharmacokinetic properties including bioavailability and catabolic potential. Affinity labeling analogs of $1,25(\text{OH})_2\text{D}_3$, that alkylate the ligand binding pocket of vitamin D receptor (VDR) can potentially avoid catabolism with the resultant effect of higher pharmacological efficacy at lower doses. $1,25$ -Dihydroxyvitamin D_3 -3-bromoacetate [$1,25(\text{OH})_2\text{D}_3$ -3-BE], a VDR-affinity alkylating analog of $1,25(\text{OH})_2\text{D}_3$ displays significantly stronger growth-inhibitory effect than the parent hormone in prostate (androgen-sensitive and androgen-insensitive), kidney, pancreas and bladder cancer cells, but not in breast, colon and lung cancer cells. $1,25(\text{OH})_2\text{D}_3$ -3-BE also shows strong tumor inhibition in a mouse xenograft model of androgen-insensitive prostate cancer. Probable mechanism of action of this VDR-alkylating analog will be discussed.

Invited Lecture:

Indian Institute of Chemical Biology, Kolkata, India, May 24, 2007

Title: Group specific component: a protein that wears multiple hats

Mechanistic and pharmacodynamic studies of a 25-hydroxyvitamin D₃ derivative in prostate cancer cells

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Abstract

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the biologically active form of vitamin D has strong antiproliferative effects in cancer cells. But it is highly toxic at therapeutic doses. We have observed that 25-hydroxyvitamin D₃-3-bromoacetate (25-OH-D₃-3-BE), a derivative of 25-hydroxyvitamin D₃, the pro-hormonal form of 1,25(OH)₂D₃ has strong growth-inhibitory and proapoptotic properties in hormone-sensitive and hormone-refractory prostate cancer cells. In the present investigation we demonstrate that the antiproliferative effect of 25-OH-D₃-3-BE is predominantly mediated by VDR in ALVA-31 prostate cancer cells. In other mechanistic studies we show that the proapoptotic property of 25-OH-D₃-3-BE is related to the inhibition of phosphorylation of Akt, a pro-survival protein. Furthermore, we carried out cellular uptake and serum stability studies of 25-OH-D₃-3-BE to demonstrate potential therapeutic applicability of 25-OH-D₃-3-BE in hormone-sensitive and hormone-insensitive prostate cancer.

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Keywords: 25-Hydroxyvitamin D₃-derivative; 1,25-Dihydroxyvitamin D₃; Prostate cancer; Vitamin D receptor; Akt pathway; Apoptosis; Serum stability; Bio-availability; Cellular uptake

Prostate cancer is the second leading cause of cancer death among men in the US. Although it mostly affects elderly men, the number of younger men with prostatic carcinoma is significant and increasing. Change in life style and increase in longevity has further emphasized the need for the effective treatment of prostate cancer, particularly those cancers that do not respond to androgen-ablation therapy [1]. The current clinical interventions for prostate cancer include surgical removal of prostate, radiation, cryotherapy and chemotherapy. However, these clinical strategies are associated with life-altering side effects including, but not limited to, incontinence and impotence. The mainstay of hormone therapy to reduce the level of testosterone and block its harmful effect in the development and growth of prostate tumor includes agents that are involved in androgen-deprivation and androgen recep-

tor antagonism. However, for prostate cancers, localized and/or metastatic, which fail to respond to androgen-ablation therapy no therapy is currently available.

Numerous epidemiological studies have demonstrated the importance of dietary vitamin D in preventing various cancers [2–4]. In addition, the therapeutic potential of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the biologically active metabolite of vitamin D, and its analogs either as monotherapy or in combination with chemotherapeutic agents in cancer is well-documented [5–13]. Although some analogs (e.g. EB-1089) have shown promise [14,15], and Calcipotriene (Dovonex) has been approved by FDA for psoriasis, availability of efficacious vitamin D-based cancer drugs with low toxicity has remained elusive.

The design, synthesis and development of non-toxic analogs of vitamin D has focused primarily on chemical modifications of various parts of 1,25(OH)₂D₃ because this dihydroxy metabolite of vitamin D₃ is biologically the most active form of the hormone. Although 25-hydroxyvitamin

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D₃ (25-OH-D₃), the non-toxic pre-hormonal form of 1,25(OH)₂D₃ has long been considered to be biologically inactive, two recent publications demonstrate considerable antiproliferative activity of this molecule in prostate and pancreatic cancer cells underscoring the potential of 25-OH-D₃ as a potential antiproliferative agent for prostate cancer therapy [16,17]. We reported that 25-hydroxyvitamin D₃-3β-(2)-bromoacetate (25-OH-D₃-3-BE), a derivative of 25-OH-D₃, shows strong antiproliferative and pro-apoptotic properties in a host of androgen-sensitive and androgen-refractory prostate cancer cells suggesting a translational potential of this compound in prostate cancer [18]. In the present study, we investigated mechanistic aspects of the growth inhibitory and pro-apoptotic properties of 25-OH-D₃-3-BE in prostate cancer cells. We also carried out cellular uptake and serum-stability analyses of this compound in view of its translational potential. A thorough understanding of the molecular mechanisms of 25-OH-D₃-3-BE in human prostate cancer cells will aid in the development of this compound as a potential chemotherapeutic agent for prostate cancer.

Materials and methods

Compounds. 25-OH-D₃-3-BE and 25-hydroxyvitamin D₃-3β-[2¹⁴C]-bromoacetate [¹⁴C-25-OH-D₃-3-BE] (sp. activity 14.3 mCi/mmol) was synthesized according to published procedures from our laboratory [19]. [25(26)-³H]-25-Hydroxyvitamin D₃-3β-bromoacetate [³H-25-OH-D₃-3-BE] (sp. activity 0.02 μCi/μmol) was synthesized by spiking a sample of 25-OH-D₃ with [25(26)-³H]-25-hydroxyvitamin D₃ (50,000 cpm, specific activity 20.6 Ci/mmol and treating the mixture with bromoacetic acid, dicyclohexylcarbodiimide and 4-*N,N'*-dimethylaminopyridine in anhydrous dichloromethane, and purifying the product by preparative thin layer chromatography on a silica plate with 25% ethyl acetate in hexanes as eluant [19].

Cell culture. ALVA-31, DU-145 and PC-3 cells were purchased from American Type Culture Collection, Manassas, VA; and were grown in RPMI 1640 or DMEM media (Gibco) containing 5% fetal bovine serum (FBS). ALVA-31 VDR-sense and VDR-antisense cells were grown in RPMI 1640 containing 5% FBS and 400 μg/mL G418 (Invitrogen).

Cellular proliferation assay. ALVA-31 human prostate cancer cells were stably transfected with an antisense VDR expression vector and an empty vector, and assayed for their response to 1,25(OH)₂D₃ or 25-OH-D₃-3-BE [20]. Antisense cells (3000 cells/well) and vector control cells (1000 cells/well) were seeded in 24 well dishes and allowed to attach for 16 h. The cells were treated with 1,25(OH)₂D₃, 25-OH-D₃-3-BE or ethanol control, and incubated for 6 days with treatment changes every 2 days. Monolayers were harvested after six days for DNA quantitation by the Hoechst 33258 fluorescence assay [21]. Triplicate determinations were used to calculate the mean DNA concentration ± standard error.

Phosphorylated Akt analysis. PC-3 cells were grown to 70–80% confluency in RPMI media containing 10% FBS in 35 mm tissue culture dishes. The media was replaced with media containing 10^{−6} M each of either 1,25(OH)₂D₃ or 25-OH-D₃-3-BE or ethanol control and allowed to incubate 24 h in a humidified 37 °C, 5% CO₂ incubator. Following the treatment, the cell monolayers were washed with 1 ml cold PBS and then lysed in 100 μl RIPA (50 mM Tris pH 7.4, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF and 1 mM Na₂VO₄) containing Roche Complete Protease Inhibitors. Cell lysates were subjected to centrifugation (13,000g, 15 min) and the clarified lysate was collected and the protein concentration determined by Bradford Assay (Bio-Rad). Electrophoresis was performed using 10% SDS-PAGE gels and 40 μg of lysate per lane followed by transfer to Immobilon

membrane (Millipore). Membrane was blocked with PBS containing 5% non-fat dry milk and 0.05% Tween-20, probed with anti-phospho-Ser473-Akt antibody and anti-Akt antibody (Cell Signaling Technologies) and detected by enhanced chemiluminescence (Perkin Elmer).

Cellular uptake of [¹⁴C]-25-OH-D₃-3-BE in DU-145 cells. DU-145 cells were grown to approximately 50% confluence in 35 mm dishes in DMEM media containing 10% FBS and additives, and incubated with [¹⁴C]-25-OH-D₃-3-BE (10,000 cpm in 10 μl of ethanol) in 1 ml of the media at 37 °C for 60 min. Following the incubation media was withdrawn and the cells were washed thoroughly (5 × 5 ml) with phosphate buffered saline (PBS). Then 5 ml of methanol was added to the plate and the cells were scraped off with a rubber policeman. The plate was washed thoroughly with 3 × 1 ml of methanol and 3 × 1 ml of PBS. Combined media and cell extracts were lyophilized and re-dissolved/suspended in 3 ml of water. The aqueous mixtures from cells and media fraction were extracted with 5 × 2 ml of ethyl acetate. The organic extract of each fraction was dried under nitrogen and re-dissolved in the mobile phase (10% H₂O-MeOH) for HPLC analysis. These extracts were analyzed by reverse phase HPLC using an Agilent 5 μm C₁₈ column, 10% H₂O in methanol mobile phase, 1.5 ml/min flow rate, 254 nm detection wave length (for the unlabeled standards) in an Agilent Series 1100 HPLC system with photo diode array detector. Effluent from the HPLC was directly introduced into a Radiomatic OnLine radioactivity detector (Radiomatic Instruments, Tampa, FL). Prior to the analysis of the organic extracts, a mixture containing a standard sample of 25-OH-D₃-3-BE was analyzed by the same system. This assay was run in duplicate.

Serum-stability of [³H]-25-OH-D₃-3-BE. A 0.5 ml aliquot of a pooled human serum sample was incubated with [³H]-25-OH-D₃-3-BE (10,000 cpm, dissolved in 10 μl of ethanol) at 37 °C for 60 min followed by extraction with 10 × 0.5 ml of ethyl acetate. The organic extracts were dried under a stream of argon, re-dissolved in mobile phase (10% H₂O in methanol) and analyzed by reverse phase HPLC as described before, except in this case fractions from HPLC were collected manually at one min intervals. The fractions were mixed with scintillation cocktail and counted for radioactivity in a scintillation counter. A solution containing standard samples of 25-OH-D₃ and 25-OH-D₃-3-BE was run in the HPLC as a standard.

Results and discussion

The antiproliferative effect of 25-OH-D₃-3-BE is mediated by VDR in ALVA-31 prostate cancer cells

In previous studies, we described that 25-OH-D₃-3-BE, a derivative of 25-OH-D₃ that affinity alkylates the hormone-binding pocket of VDR [22], strongly inhibits the growth of several androgen-sensitive and androgen insensitive prostate cancer cells via induction of apoptotic pathways [18]. We also demonstrated that 25-OH-D₃-3-BE induces 1α,25-dihydroxyvitamin D₃-24-hydroxylase (24-OHase) promoter activity, and promotes strong interaction between VDR and general transcriptional factors RXR and GRIP-1 [18]. These results suggested that the cellular activities of 25-OH-D₃-3-BE are similar to those of 1,25(OH)₂D₃, and mediated by a VDR-activation pathway.

To confirm that the growth inhibitory properties of 25-OH-D₃-3-BE are mediated by its interaction with VDR, we performed cellular proliferation assays in ALVA-31 “VDR-null” prostate cancer cells. We argued that since growth inhibitory effects of 1,25(OH)₂D₃ is manifested via its interaction with VDR in ALVA-31 cells [20], if the antiproliferative effects of 25-OH-D₃-3-BE is also modulated through VDR, we can expect that 25-OH-D₃-3-BE-mediated

growth inhibition of ALVA-31 cells would be either eliminated or diminished in cells transfected with a VDR-anti-sense vector.

As shown in Fig. 1 growth of VDR-sense cells (empty vector) is strongly inhibited by 10^{-7} M of $1,25(\text{OH})_2\text{D}_3$ as reported earlier [20]. Conversely, the growth of antisense cells treated with 10^{-7} M of $1,25(\text{OH})_2\text{D}_3$ is similar to that of ethanol-control, confirming the requirement of VDR in the antiproliferative activity of $1,25(\text{OH})_2\text{D}_3$ in ALVA-31 cells. In the case of 25-OH-D₃-3-BE, 10^{-6} M of this compound strongly inhibited the growth of empty vector (sense cells), while growth of anti-sense cells is similar to that of ethanol control. However, with 10^{-7} M of 25-OH-D₃-3-BE, the growth of both sense and antisense cells are similar to that of the control. This result is in accordance with our previous studies where we observed the antiproliferative effect of 25-OH-D₃-3-BE is strongest at 10^{-6} M dose, and decreased significantly at lower doses [18]. Overall, the result of this assay strongly emphasizes the requirement for VDR in mediating the antiproliferative effect of 25-OH-D₃-3-BE in prostate cancer cells.

We observed that 25-OH-D₃-3-BE is approximately one log scale less efficient than $1,25(\text{OH})_2\text{D}_3$ in inhibiting the growth of wild type ALVA-31. Earlier we reported similar dose-dependence (of 25-OH-D₃-3-BE) in modulating the message for 24-OHase and inducing interaction of VDR with RXR GRIP-1 transcription factors [18]. Differences in the potency of vitamin D analogs to induce various gene-regulatory events through VDR have been reported. For example, 2MD, an analog of $1,25(\text{OH})_2\text{D}_3$ shows a range of sensitivity for regulating gene expression from

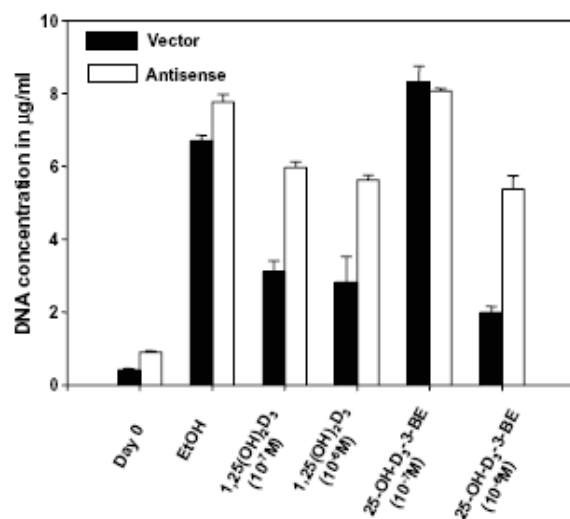


Fig. 1. 25-OH-D₃-3-BE inhibits the growth of ALVA-31 prostate cancer cells through a VDR-dependent mechanism. ALVA-31 control (vector) and ALVA-31 VDR “null” cells (antisense) were treated with the indicated doses of $1,25(\text{OH})_2\text{D}_3$, 25-OH-D₃-3-BE and ethanol control for 6 days. Monolayers were harvested for DNA quantification. Each condition was conducted in triplicate. Values are presented as mean DNA concentration $\pm$ standard error.

$\text{ED}_{50} = 10^{-11}$ mol/L for the up-regulation of RANKL to $\text{ED}_{50} > 10^{-10}$ mol/L for induction of osteopontin and 24-OHase in mouse osteoblasts [23]. Similarly, it was shown that RO-26-9228, an analog of $1,25(\text{OH})_2\text{D}_3$ has an ED_{50} of 2.1×10^{-8} mol/L for the induction of 24-OHase, and an ED_{50} of 2.7×10^{-7} mol/L for the induction of Calbindin D9K in Caco-2 cells [24]. Therefore, the lower efficacy of 25-OH-D₃-3-BE compared with $1,25(\text{OH})_2\text{D}_3$ in modulating gene-regulatory events is not unexpected.

25-OH-D₃-3-BE inhibits Akt phosphorylation in PC3 prostate cancer cells

Previously, we reported that 25-OH-D₃-3-BE induced nuclear DNA-fragmentation and activated caspases 3, 8 and 9, hallmarks of apoptosis, in PC3 cells while an equimolar concentration of $1,25(\text{OH})_2\text{D}_3$ and 25-OH-D₃ failed to do so [18]. Induction of caspases and fragmentation of nuclear DNA represent downstream signaling markers of apoptosis and these markers are regulated by their upstream modulators such as Akt kinase. We postulated that induction of apoptosis by 25-OH-D₃-3-BE might be mediated by the down-regulation of Akt-activity resulting in the observed up-regulation of pro-apoptotic proteins.

Akt (*aka* protein kinase B, PKB) is a serine/threonine kinase that is involved in signal transduction by phosphoinositol-3'-kinase/Akt pathway. Akt is involved in a variety of normal cellular functions. In addition, Akt has profound effects in tumorigenesis, cell proliferation, growth and survival. Recently it has been shown that Akt regulates G(1) cell cycle progression and cyclin expression in prostate cancer cells [25]. Another study showed upregulation of Akt and other growth promoting signaling molecules in malignant prostate epithelial cells [26]. We postulated that induction of apoptosis by 25-OH-D₃-3-BE might be mediated by the down-regulation of Akt-activity resulting in the up-regulation of pro-apoptotic proteins. As shown in Fig. 2, we observed significant inhibition of phosphorylated Akt in prostate cancer cells treated with 25-OH-D₃-3-BE, while Akt phosphorylation was unaffected by $1,25(\text{OH})_2\text{D}_3$.

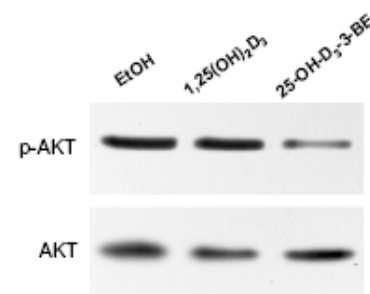


Fig. 2. 25-OH-D₃-3-BE inhibits Akt phosphorylation in PC-3 prostate cancer cells. PC-3 cells were treated with equimolar concentrations of $1,25(\text{OH})_2\text{D}_3$, 25-OH-D₃-3-BE and ethanol control and Western analysis performed for phosphorylated Akt (p-Akt). The blot was stripped and re-probed for total Akt to ensure equal loading of protein in the lanes.

These results suggested that 25-OH-D₃-3-BE may exert its antiproliferative effects, at least in part, by inhibiting this pro-survival pathway.

25-OH-D₃-3-BE is taken up in its intact form by DU-145 cells

The antiproliferative and apoptotic activities of 25-OH-D₃-3-BE in prostate cancer cells strongly endorse its poten-

tial as a therapeutic agent for prostate cancer. However, evaluation of this potential requires examination of its pharmacodynamic properties, including its bio-availability and stability in serum.

25-OH-D₃-3-BE contains a hydrolytically unstable ester bond and its hydrolysis would produce equivalent amounts of 25-OH-D₃ and bromoacetic acid. In an earlier study we demonstrated that the growth inhibitory property of 25-OH-D₃-3-BE is related strictly to the intact molecule

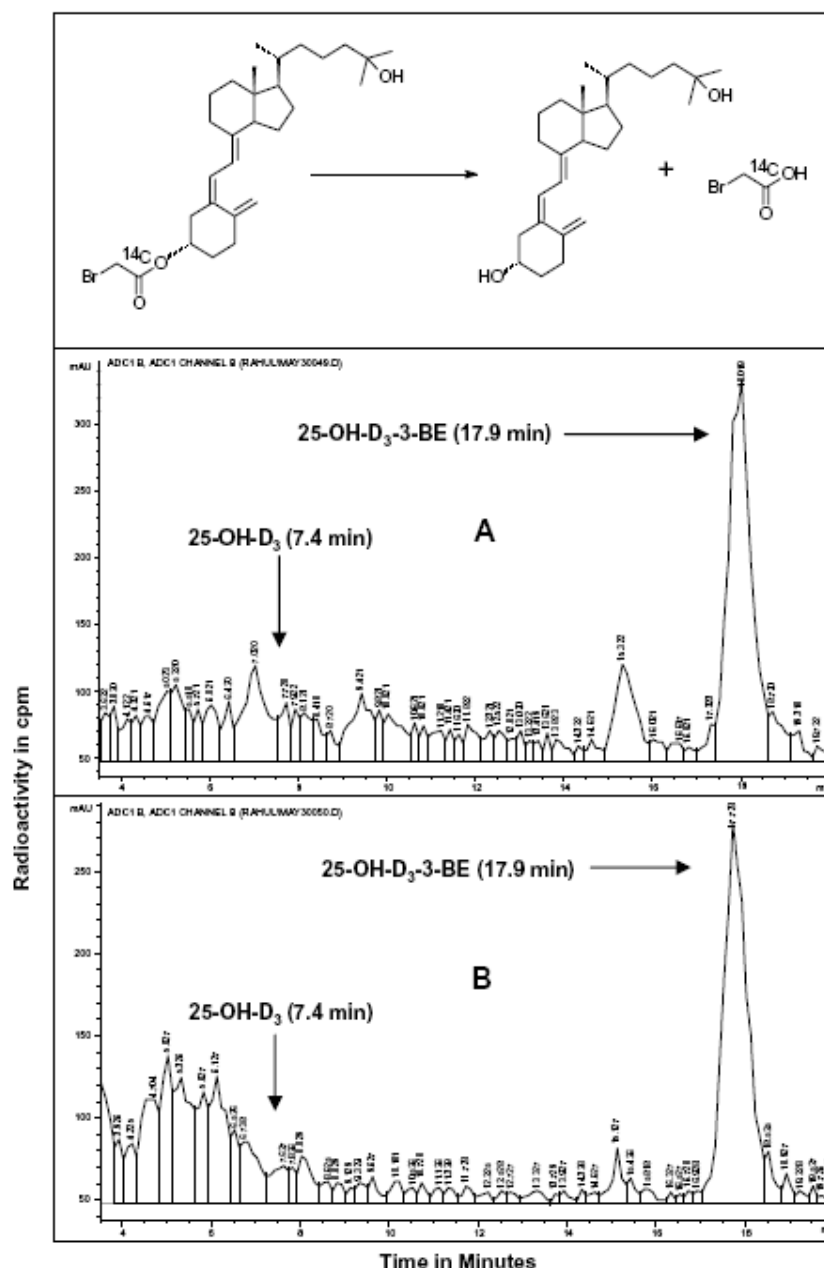


Fig. 3. 25-OH-D₃-3-BE is taken up by DU-145 prostate cancer cells in its intact form. DU-145 prostate cancer cells were treated with ¹⁴C-25-OH-D₃-3-BE and HPLC analysis performed on the media (A) and whole cell extracts (B). Fractions were counted for radioactive content. Hydrolysis of ¹⁴C-25-OH-D₃-3-BE producing unlabeled 25-OH-D₃ and ¹⁴C-bromoacetic acid is shown in the top of the figure. (A) Media extract; (B) Cellular extract. Position of the 25-OH-D₃ peak (retention time 7.4 min) is shown with an arrow.

and its hydrolysis products [18]. Therefore, we carried out a cellular uptake study of 25-OH-D₃-3-BE in DU-145 cells. The goal of this study was to determine whether we can isolate 25-OH-D₃-3-BE in its intact form from cellular extracts. For this study we employed a radiolabeled version of 25-OH-D₃-3-BE, i.e. 25-hydroxyvitamin D₃-3β-[2-¹⁴C]-bromoacetate (¹⁴C-25-OH-D₃-3-BE). We argued that hydrolysis of ¹⁴C-25-OH-D₃-3-BE should produce unlabeled 25-OH-D₃ and radiolabeled bromoacetic acid (¹⁴C-bromoacetic acid) (Top panel, Fig. 3). Bromoacetic acid is a polar molecule and therefore it will not be extracted from the media and cellular extracts by an organic solvent. Therefore, the presence of a radioactive peak corresponding to 25-OH-D₃-3-BE would represent intact 25-OH-D₃-3-BE.

HPLC analysis of the organic extracts of media and DU-145 cells incubated with ¹⁴C-25-OH-D₃-3-BE demonstrate that chromatograms of both media (Fig. 3A) and cellular fractions (Fig. 3B) contain a single well-defined peak at 17.9 min representing ¹⁴C-25-OH-D₃-3-BE (Fig. 3, mid-

dle and bottom Panels). There are low-level and unresolved polar peaks in media and cellular extracts, particularly in the cellular extract, possibly representing alkylated small molecules derived from the buffer (alkylated proteins are usually not extracted from aqueous phase by an organic solvent, like ethyl acetate used in this study). Collectively these results strongly suggest that 25-OH-D₃-3-BE is taken up by the cells in its intact form.

25-OH-D₃-3-BE is stable in human serum

Serum-stability is an important aspect of a potential therapeutic agent, because it determines the availability of the molecule in its intact and bioactive form. This study required that we incubate human serum with 25-OH-D₃-3-BE and then carry out an organic solvent extraction and HPLC-analysis of the extract. We argued that ¹⁴C-25-OH-D₃-3-BE, used for the previous study, could not be used here, because hydrolysis of ¹⁴C-25-OH-D₃-3-BE would result in a loss of radioactivity (as ¹⁴C-bromoacetic

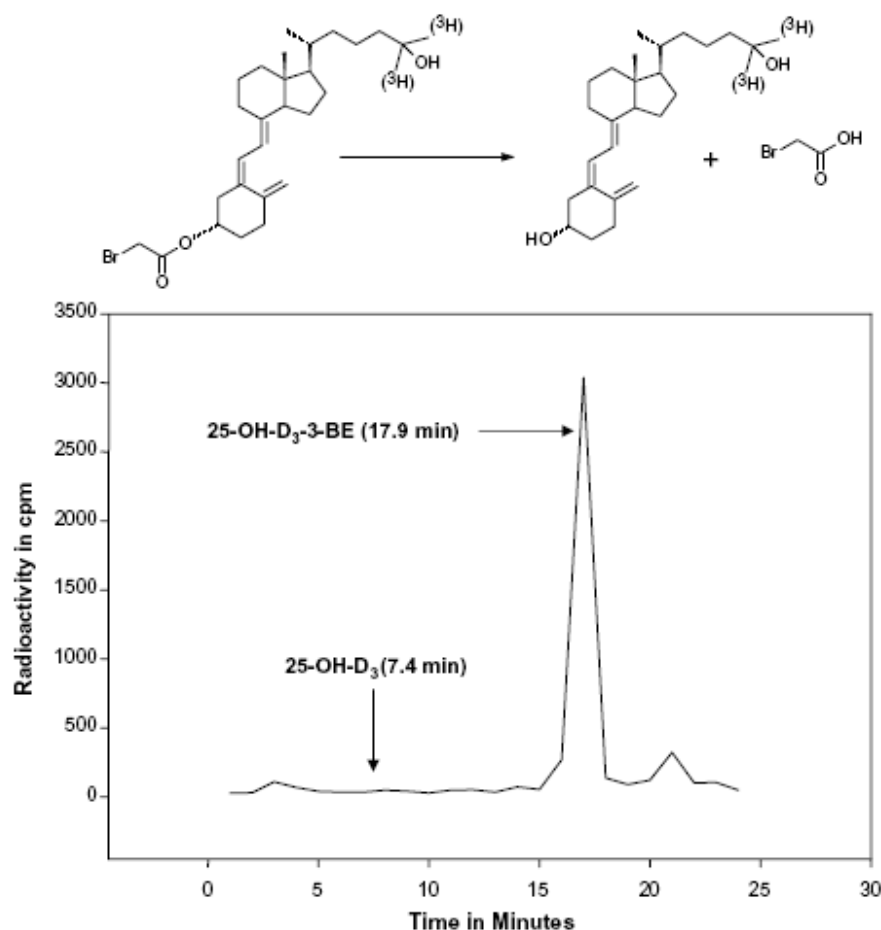


Fig. 4. 25-OH-D₃-3-BE is stable in human serum. ³H-25-OH-D₃-3-BE was synthesized and incubated with human serum, followed by extraction with an organic solvent, and HPLC analysis of the organic extract. Fractions from HPLC were mixed with scintillation cocktail and counted for radioactivity. (Upper panel) Representation of the hydrolysis of ³H-25-OH-D₃-3-BE leading to the production of ³H-25-OH-D₃ and unlabeled bromoacetic acid. (Lower panel) Peak indicating intact ³H-25-OH-D₃-3-BE. Position of the 25-OH-D₃ peak (retention time 7.4 min) is shown with an arrow.

acid) in the aqueous phase. Therefore, in order to determine the extent of hydrolysis of 25-OH-D₃-3-BE in serum we synthesized ³H-25-OH-D₃-3-BE in which the radiolabel (³H) is in the 25-OH-D₃ moiety. Hence, its hydrolysis would produce ³H-25-OH-D₃ and unlabeled bromoacetic acid (as noted in Fig. 4, inset); and the organic extract will thus contain a combination of ³H-25-OH-D₃ and ³H-25-OH-D₃-3-BE if hydrolysis occurs.

The results of this assay (Fig. 4) show that the majority of radioactivity is concentrated in a single peak corresponding to ³H-25-OH-D₃-3-BE. The absence of a radioactive peak corresponding to ³H-25-OH-D₃ (hydrolysis product) indicates that ³H-25-OH-D₃-3-BE is fully stable under these experimental conditions. Absence of hydrolysis also suggests that 25-OH-D₃-3-BE maintains considerable bio-availability in its intact form to attest its potential as a therapeutic agent for prostate cancer.

In summary, results of the studies described herein strongly suggest that the growth inhibitory properties of 25-OH-D₃-3-BE are mediated by a VDR-dependent pathway similar to the native hormone, 1,25(OH)₂D₃, while its apoptotic property is manifested via activation of Akt-phosphorylation pathway. Additionally, results included in this communication demonstrate favorable pharmacodynamic properties, including cellular uptake in intact form and serum-stability leading to the belief that 25-OH-D₃-3-BE can potentially be developed as a therapeutic agent for prostate cancer.

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References

- [1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, C. Smigal, M.J. Thun, Cancer Statistics 2006. CA, Cancer J. Clin. 56 (2) (2006) 106–130.
- [2] E. Giovannucci, Y. Liu, E.B. Rimm, B.W. Hollis, C.S. Fuchs, M.J. Stampfer, W.C. Willett, Prospective study of predictors of vitamin D status and cancer incidence and mortality in men, J. Natl. Cancer Inst. 98 (2006) 451–459.
- [3] E. Giovannucci, Y. Liu, M.J. Stampfer, W.C. Willett, A prospective study of calcium intake and incident and fatal prostate cancer, Cancer Epidemiol. Biomarkers Prev. 15 (2006) 203–210.
- [4] E.K. Wei, E. Giovannucci, C.S. Fuchs, W.C. Willett, C.S. Mantzoros, Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study, J. Natl. Cancer Inst. 97 (2005) 1688–1694.
- [5] S. Matsuda, G. Jones, Promise of vitamin D analogues in the treatment of hyperproliferative conditions, Mol. Cancer Ther. 5 (2006) 797–808.
- [6] D.L. Trump, J. Muindi, M. Fakih, W.D. Yu, C.J. Johnson, Vitamin D compounds: clinical development as cancer therapy and prevention agents, Anticancer Res. 26 (2006) 2551–2556.
- [7] A.L. Sutton, P.N. MacDonald, Vitamin D: more than a “bone-a-fide” hormone, Mol. Endocrinol. 17 (2003) 777–791.
- [8] M.G. Fakih, D.L. Trump, J.R. Muindi, J.D. Black, R.J. Bernardi, P.J. Creaven, J. Schwartz, M.G. Brattain, A. Hutson, R. French, C.S. Johnson, A phase I pharmacokinetic and pharmacodynamic study of intravenous calcitriol in combination with oral gefitinib in patients with advanced solid tumors, Clin. Cancer Res. 13 (2007) 1216–1223.
- [9] D.L. Trump, D.M. Potter, J. Muindi, A. Brufsky, C.S. Johnson, Phase II trial of high-dose, intermittent calcitriol (1,25 dihydroxyvitamin D₃) and dexamethasone in androgen-independent prostate cancer, Cancer 106 (2006) 2136–2142.
- [10] J.R. Muindi, Y. Peng, D.M. Potter, P.A. Hershsberger, J.S. Tauch, M.J. Capozzoli, M.J. Egorin, C.S. Johnson, D.L. Trump, Pharmacokinetics of high-dose oral calcitriol phase I trial of calcitriol and paclitaxel, Clin. Pharmacol. Ther. 72 (2003) 648–659.
- [11] P.A. Hershsberger, W.D. Yu, R.A. Modzelewski, R.M. Rueger, C.S. Johnson, D.L. Trump, Calcitriol (1,25-dihydroxycholecalciferol) enhances paclitaxel antitumor activity *in vitro* and *in vivo* and accelerates paclitaxel-induced apoptosis, Clin. Cancer Res. 7 (2001) 1043–1051.
- [12] W.D. Yu, M.C. McElwain, R.A. Modzelewski, D.M. Russell, D.C. Smith, D.L. Trump, C.S. Johnson, Enhancement of 1,25-dihydroxyvitamin D₃-mediated antitumor activity with dexamethasone, J. Natl. Cancer Inst. 90 (1998) 34–41.
- [13] D.C. Smith, C.S. Johnson, C.C. Freeman, J. Muindi, J.W. Wilson, D.L. Trump, A phase I trial of calcitriol (1,25-dihydroxycholecalciferol) in patients with advanced malignancy, Clin. Cancer Res. 5 (1999) 1339–1345.
- [14] K. Dalhoff, J. Dancey, L. Astrup, T. Skovsgaard, K.J. Hamberg, F.J. Loft, O. Rosmorduc, S. Erlinger, J. Bach Hansen, W.P. Steward, T. Skov, F. Burchard, T.R. Evans, A phase II study of the vitamin D analogue Seocalcitol in patients with inoperable hepatocellular carcinoma, Br. J. Cancer 89 (2003) 252–257.
- [15] T.R. Evans, K.W. Colston, F.J. Loft, D. Cunningham, D.A. Anthony, H. Gogas, J.S. de Bono, K.J. Hamberg, T. Skov, J.L. Mansi, A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer, Br. J. Cancer 86 (2002) 680–685.
- [16] G.G. Schwartz, D. Eads, A. Rao, S.D. Cramer, M.C. Willingham, T.C. Chen, D.P. Jamieson, L. Wang, K.L. Burnstein, M.F. Holick, C. Koumenis, Pancreatic cancer cells express 25-hydroxyvitamin D-1 alpha-hydroxylase and their proliferation is inhibited by the prohormone 25-hydroxyvitamin D₃, Carcinogenesis 25 (2004) 1015–1026.
- [17] 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.
- [18] N. Swamy, T.C. Chen, S. Peleg, P. Dhawan, S. Christakos, L.V. Stewart, N. Weigel, R.G. Mehta, M.F. Holick, R. Ray, Inhibition of proliferation and induction of apoptosis by 25-hydroxyvitamin D₃-3β-(2)-bromoacetate, a non-toxic and vitamin D receptor-alkylating analog of 25-hydroxyvitamin D₃ in prostate cancer cells, Clin. Cancer Res. 10 (2004) 8018–8027.
- [19] N. Swamy, R. Ray, Affinity labeling of rat serum vitamin D-binding protein, Arch. Biochem. Biophys. 333 (1996) 139–144.
- [20] T.E. Hedlund, K.A. Moffatt, G.J. Miller, Vitamin D receptor expression is required for growth modulation by 1 alpha,25-dihydroxyvitamin D₃ in the human prostatic carcinoma cell line ALVA-31, J. Steroid Biochem. Mol. Biol. 58 (1996) 277–288.
- [21] C. Labarca, K. Paigen, A simple, rapid, and sensitive DNA assay procedure, Anal. Biochem. 102 (1980) 344–352.
- [22] N. Swamy, J. Addo, R. Ray, Development of an affinity-driven double cross-linker: isolation of a ligand-activated factor, associated with vitamin D receptor-mediated transcriptional machinery, Bioorg. Med. Chem. Lett. 10 (2000) 361–364.
- [23] N.K. Shevde, L.A. Plum, M. Clagett-Dame, H. Yamamoto, J.W. Pike, A potent analog of 1-alpha,25-dihydroxyvitamin D₃ selectively induces bone formation, Proc. Natl. Acad. Sci. USA 99 (2002) 13487–13491.

- [24] A. Ismail, C.V. Nguyen, A. Ahene, J.C. Fleet, M.R. Uskokovic, S. Peleg, Effect of cellular environment on the selective activation of the vitamin D receptor by 1- α ,25-dihydroxyvitamin D₃ and its analog 1- α -fluoro-16-ene-20-epi-23-ene-26,27-bishomo-25-hydroxyvitamin D₃ (Ro-26-9228), *Mol. Endocrinol.* 18 (2004) 393–411.
- [25] N. Gao, Z. Zhang, B.H. Jiang, X. Shi, Role of PI3K/Akt/mTOR signaling in the cell cycle progression of human prostate cancer, *Biochem. Biophys. Res. Commun.* 310 (2003) 1124–1132.
- [26] A.R. Uzgaré, J.T. Isaacs, Enhanced redundancy of in Akt and mitogen-activated protein kinase induced survival of malignant versus normal prostate epithelia, *Cancer Res.* 64 (2004) 6190–6199.