Inhibition of Proliferation and Induction of Apoptosis by 25-Hydroxyvitamin D3-3β-(2)-Bromoacetate, a Nontoxic and Vitamin D Receptor-Alkylating Analog of 25-Hydroxyvitamin D3 in Prostate Cancer Cells

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ABSTRACT

The 25-hydroxyvitamin D3 (25-OH-D3) is a nontoxic and low-affinity vitamin D receptor (VDR)-binding metabolic precursor of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]. We hypothesized that covalent attachment of a 25-OH-D3 analog to the hormone-binding pocket of VDR might convert the latter into transcriptionally active holo-form, making 25-OH-D3 biologically active. Furthermore, it might be possible to translate the nontoxic nature of 25-OH-D3 into its analog. We showed earlier that 25-hydroxyvitamin D3-3-bromoacetate (25-OH-D3-3-BE) alkylated the hormone-binding pocket of VDR. In this communication we describe that 10−4 mol/L of 25-OH-D3-3-BE inhibited the growth of keratinocytes, LNCaP, and LAPC-4 androgen-sensitive and PC-3 and DU145 androgen-refractory prostate cancer cells, and PZ-HPV-7 immortalized normal prostate cells with similar or stronger efficacy as 1,25(OH)2D3. But its effect was strongest in LNCaP, PC-3, LAPC-4, and DU145 cells. Furthermore, 25-OH-D3-3-BE was toxic to these prostate cancer cells and caused these cells to undergo apoptosis as shown by DNA-fragmentation and caspase-activation assays. In a reporter assay with COS-7 cells, transfected with a 1α,25-dihydroxyvitamin D3-24-hydroxylase (24-OHase)-construct and VDR-expression vector, 25-OH-D3-3-BE induced 24-OHase promoter activity. In a“pull down assay” with PC-3 cells, 25-OH-D3-3-BE induced strong interaction between VDR and general transcription factors, retinoid X receptor, and GRIP-1. Collectively, these results strongly suggested that the cellular effects of 25-OH-D3-3-BE were manifested via 1,25(OH)2D3/VDR signaling pathway. A toxicity study in CD-1 mice showed that 166 μg/kg of 25-OH-D3-3-BE did not raise serum-calcium beyond vehicle control. Collectively, these results strongly suggested that 25-OH-D3-3-BE has a strong potential as a therapeutic agent for androgen-sensitive and androgen-refractory prostate cancer.

INTRODUCTION

Alkylating agents, such as estramustine, lumustine, procarbazine, busulfan, cyclophosphamide, and chlorambucil, platinum coordination complexes are important components in the standard cancer chemotherapeutic regimen. However, majority of these drugs are nonspecific and produce significant to severe side effects, particularly at doses required for the reduction/elimination of tumor (1). Affinity alkylating compounds, on the other hand, cross-link to the substrate/ligand-binding sites of target enzymes/receptors; thus, they can potentially modulate the biological property associated only with the target molecule/molecules (2). We postulated that such target specificity might lower the therapeutic dose of the compounds and can potentially avoid harmful side effects.

Vitamin D receptor (VDR), the nuclear receptor for the vitamin D hormone, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] is a known target for the potential development of anticancer drugs (3–5). The main obstacle in such efforts has been toxicity of 1,25(OH)2D3, and many of its synthetic analogs related to hypercalcemia, particularly at doses to have a beneficial effect. The cell-regulatory properties of 1,25(OH)2D3, and its synthetic analogs are associated with the activation of VDR, but a similar link with calcemic activity is yet to be established firmly. A robust effort has been underway to develop vitamin D derivatives with strong anti-proliferative property and reduced toxicity. This effort has produced many vitamin D analogs; and it has been possible to dissociate, at least in part, hypercalcemia from antiproliferative properties in certain analogs, classified as “noncalcemic vitamin D analogs” (6). EB-1089, one such analog, is currently in clinical trials for breast, colorectal, pancreatic, and hepatocellular carcinomas (7–11). Such success has provided a strong impetus to addi-
tionally develop therapeutically important vitamin D analogs for a broad range of diseases, including cancer.

The 25-hydroxyvitamin D3 (25-OH-D3), the metabolic precursor of 1,25(OH)2D3, has a significantly reduced VDR-binding affinity. As a result, 25-OH-D3 is not considered to be biologically active. Additionally, it is nontoxic [serum concentration of 25-OH-D3 is 40 to 100 ng/mL versus 8 to 10 pg/mL for 1,25(OH)2D3]. We hypothesized that if 25-OH-D3 could be covalently attached to the hormone-binding pocket of apo-VDR, it might be possible to convert the latter into transcriptionally active holo-form. This would make 25-OH-D3 biologically active. Furthermore, it might be possible to translate the nontoxic nature of 25-OH-D3 into its VDR-alkylating analog. Recently, we showed that 25-hydroxyvitamin D3-3β-(2-bromoacetoacetate (25-OH-D3-3-BE), a derivative of 25-OH-D3, specifically alkylated the hormone-binding pocket of VDR (12). Therefore, 25-OH-D3-3-BE became an ideal candidate to validate our hypothesis.

In the present study, we investigated the effect of 25-OH-D3-3-BE in a set of normal and malignant cell lines and observed that antiproliferative property of 25-OH-D3-3-BE was most pronounced in prostate cancer cells. In addition, we observed that 25-OH-D3-3-BE caused apoptosis in prostate cancer cells; an observation supported by DNA fragmentation and caspase-activation studies. Mechanistic studies showed that the effects of 25-OH-D3-3-BE were mediated by VDR. Moreover, in a CD-1 mouse model, it was observed that 25-OH-D3-3-BE did not raise serum calcium beyond control at doses considered to be highly toxic for 1,25(OH)2D3 and many of its synthetic analogs. Results of these studies and their implications are discussed in this communication.

MATERIALS AND METHODS

The 25-OH-D3-3-BE was synthesized according to our published procedure (13). The majority of the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless mentioned otherwise. The hVDR expression vector pAVhVDR was a generous gift from Dr. Wesley Pike (University of Wisconsin, Madison, WI). All of the cell lines were obtained from American Type Culture Collection (Manassas, VA), except LAPC4 cells that were obtained from the laboratory of Charles Sawyer (Department of Medicine, University of California at Los Angeles, Los Angeles, CA).

Male CD-1 mice 6 to 8 weeks old, average weight 30 g were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in cages of five (5) in a group and were fed rat chow and water ad lib. Animal experiment was carried out in the animal facility of Boston University School of Medicine with strict adherence to the guidelines of Laboratory Animal Safety Committee. Serum calcium values in blood samples were determined at the Core Chemistry Laboratory of Boston University Medical Center.

Cell Culture. PZ-HPV-7 cells were grown in MCDB media containing pituitary extract, epidermal growth factor, and 1% penicillin/streptomycin. Keratinocytes were also grown in the same media with additional PG1 and insulin. PC-3, LNCaP, and DU-145 cells were grown in RPMI containing 10% fetal bovine serum (FBS) and antibiotics. MCF-7 cells were grown in DMEM containing 10% FBS and antibiotics. LAPC-4 cells were maintained in IMEM containing antibiotics including 1% t-glutamine and 10 mmol/L of R1881, a synthetic progestin. MC3T3 cells were grown in αMEM containing 10% FBS and antibiotics. In general, cells were grown in 35-mm dishes to 70 to 80% confluence and then plated into 24-well plates in respective media. After the cells grew to ~70% confluence, they were serum-starved for 20 hours (PC-3, LNCaP, and DU-145 cells) followed by incubation with steroid samples. Keratinocytes and PZ-HPV-7 cells, after reaching 70% confluence, were kept in MCDB media without additives for 20 hours before treatment with steroids. In general, reagents were dissolved in EtOH, and dilution with the media was adjusted in such a way that concentration of EtOH was 0.1% v/v.

In a separate experiment (cell counting), LAPC-4, LNCaP, MCF-7, and MC3T3 cells were grown to desired confluence and treated with the reagents (without serum starvation) for 24 hours (LNCaP, MC3T3, and LAPC-4) or 48 hours (MCF-7) with EtOH vehicle or 25-OH-D3-3-BE (10-6 mol/L) or 1,25(OH)2D3 (10-7 mol/L). At the end of the experiment, cells were detached with trypsin-EDTA and counted in a Coulter counter.

Keratinocytes, procured from neonatal foreskin after overnight trypsinization at 4°C and treatment with 0.2% EDTA, were grown in culture with a modification of the published method (14). The 3T3 cells were plated at 104 cells/35-mm tissue-culture dish and were irradiated lethally after 2 days with a 60Co source (5,000 rads). Keratinocytes, in 1 mL serum-free medium, were plated on lethally irradiated 3T3 cells. When these cells reached ~70% confluence, they were plated onto 24-well plates. Each experiment was done on primary or secondary keratinocyte cultures obtained from different skin samples.

The [3H]Thymidine Incorporation Assay. In a typical assay, cells were grown to 60 to 70% confluence in 24-well plates in respective media containing 10% FBS, and serum starved for 20 hours, followed by treatment with various agents (in 0.1% ethanol solution or EtOH vehicle) for 16 to 18 hours. 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 3 hours at 37°C. After this period, media was removed by aspiration, and the cells were washed thoroughly (×3 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 scintillation counter.

Majority of these assays were carried out in six (6) replicates with 10-7 to 10-6 mol/L of reagents. In the dose-response study, PC-3 cells were incubated with EtOH or 10-7 to 10-6 mol/L of 25-OH-D3-3-BE or 1,25(OH)2D3 for 18 hours followed by [3H]thymidine incorporation assay described above.

The 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Cell Viability Assay. LNCaP, PC-3, and DU145 cells were plated in 96-well plates (7,500 cells per well), grown overnight in DMEM (with 10% FBS and antibiotics), and serum deprived for 24 hours.
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The cells were then treated with either EtOH or 1,25(OH)2D3 (10⁻⁶ mol/L) or 25-OH-D₃-3-BE (10⁻⁶ mol/L) for 18 hours in complete media. Cell viability was measured with the CellTitre 96 AQueous Assay (Promega, Madison, WI). This assay used the tetrazolium compound (MTS, inner salt) and the electron-coupling reagent, phenazine methosulfate (15). This assay measured dehydrogenase enzyme activity found in metabolically active cells, which reduced MTS into soluble and colored formazan product, absorbance of which was measured at 490 nm. Because the production of formazan was proportional to the number of living cells, absorbance was a measure of cell-viability.

**DNA-Fragmentation Analysis.** PC-3 cells (2 × 10⁶) were treated with 0.25 × 10⁻⁶ mol/L of 1,25(OH)₂D3, 25-OH-D₃, or 25-OH-D₃-3-BE for 10 hours. Then the cells were harvested and lysed in 0.5 mL of lysis buffer (20 mmol/L Tris-HCl, 10 mmol/L EDTA, 0.5% Triton X-100 (pH 8.0)), and DNA was extracted with phenol-chloroform procedure. The extracted DNA was resuspended in 0.1 mL of 20 mmol/L Tris-HCl (pH 8), and treated with RNase, followed by electrophoresis on a 1.2% agarose gel in TAE buffer. DNA bands were visualized under UV light after ethidium bromide staining.

**Caspase Activity.** Caspase-3, -8, and -9 assays were done with Caspase colorimetric assay kit from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions. Briefly, PC3 cells (1 × 10⁶) were treated with 0.01 × 10⁻⁶ mol/L of 1,25(OH)₂D₃, 25-OH-D₃, or 25-OH-D₃-3-BE for 14 hours in culture medium (DMEM, 10% FBS, and antibiotics). The cells were collected by centrifugation at 1,000 rpm for 5 minutes. The cell pellet was lysed with lysis buffer, and the lysate was incubated on ice for 10 minutes and centrifuged at 10,000 rpm for 5 minutes. Protein was estimated with Bradford protein estimation kit (Bio-Rad Laboratories, Hercules, CA).

The enzymatic reactions were carried out in a 96-well plate. For each reaction, 100 μg lystate protein in 50 μL total volume was incubated with 50 μL of 2 × reaction buffer and 5 μL of caspase 3, caspase 8, or caspase 9 colorimetric substrates for 2 hours at 37°C. The absorbance was determined at 405 nm.

**Induction of 1α,25-Dihydroxyvitamin D₃-24-Hydroxylase (24-OHase) Promoter Activity by 25-OH-D₃-3-BE and 1,25(OH)₂D₃ in COS-7 Cells**

**Cell Transfections.** Promoter constructs containing the rat 24-OHase promoter (−1,367/+74) linked to the chloramphenicol acetyltransferase (CAT) reporter gene were used for the experiment. COS-7 cells that were transfected with the hVDR expression vector pAVhVDR. All of the transfections were done with the calcium phosphate DNA precipitation method. The COS-7 cells were seeded with 1 × 10⁵ cells/100 mm² tissue culture plate in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and allowed to grow for 18 to 20 hours or to 70 to 80% confluency. The DNA to be transfected was EtOH-preincubated. For each plate to be transfected, 450 μL of sterile ddH₂O and 50 μL of 2.5 mol/L CaCl₂ were added to the DNA pellet. This mixture was then added to 500 μL of 2 × HEPES buffer per sample dropwise while mixing. After the two solutions were combined, the resulting mixture was vortexed and allowed to sit at room temperature for 20 minutes to allow the DNA to precipitate. Finally, the DNA precipitate was mixed thoroughly, and 1 mL aliquots were added to each plate. Sixteen hours post transfection, cells were “shocked” for 1 minutes with PBS containing 10% dimethylsulfoxide, washed with PBS, and the DMEM supplemented with 2% of charcoal dextran-treated PBS was added to each plate. The cells were then treated with various doses of 1,25(OH)₂D₃ or 25 OH-D₃-3-BE for 24 hours.

**CAT Assay.** Treated cells were harvested by trypsinization for about 2 minutes at 37°C, pelleted, washed with PBS, resuspended in 0.25 mol/L Tris-HCl (pH 8.0), and lysed by freezing and thawing five (5) times. Cellular extracts were collected and used for CAT assays.

CAT analysis was done by standard protocols on the cell extracts normalized to total protein content. Fifty microliters aliquots of cellular extracts containing equal amounts of protein were combined with 25 μL of 1 mol/L Tris-HCl (pH 8.0), 53 μL of ddH₂O, 20 μL of 4 mmol/L acetyl CoA, 2 μL of 14C chloramphenicol (50 mCi/mmol; Sigma, St. Louis, MO), and 0.25 mmol/L Tris-HCl (pH 8.0) to a final volume of 150 μL. The reactions were carried out at 37°C for about 2 hours and stopped by adding 1 mL of ethyl acetate and vortexing. The samples were centrifuged at 14,000 rpm at 4°C for 10 minutes, and the upper ethyl acetate layer was removed to a microcentrifuge tube and dried under vacuum for 45 minutes. The samples were resuspended in 25 μL of ethyl acetate and spotted on a TLC plate. Chromatography was done in a chromatography chamber containing 100 mL of chloroform-methanol (95:5) for 40 minutes. The plate was dried and exposed to Kodak autoradiographic film. The resulting autoradiogram was analyzed by densitometric scanning with the Shimadzu CS9000U Dual-wavelength Flying Spot Scanner (Shimadzu Scientific Instruments, Princeton, NJ).

**Pull Down Assays to Determine the Interaction of VDR with Retinoid X Receptor (RXR) and GRIP-1 in the Presence of 1,25(OH)₂D₃ or 25-OH-D₃-3-BE in PC-3 Cells.** In this assay, PC-3 cells were incubated for either 1 or 24 hours with the indicated concentrations of 1,25(OH)₂D₃ or 25-OH-D₃-3-BE, and then the cells were scraped, homogenized, and whole-cell extracts were prepared in NETND buffer [100 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 7.8), 0.2% NP40, and 1 mmol/L dithiothreitol] containing 0.3 mol/L KCl. Then, 5 μg of purified glutathione S-transferase (GST) fusion protein (GST-GRIP or GST-RXR), and 20 μL of glutathione-Sepharose beads were added, and the volume was brought up to 100 μL with the same buffer. These mixtures were incubated for 1 or 24 hours at 4°C, and the beads were washed 3 times with 0.2 mL of NETND buffer. The bound proteins were eluted from the packed beads by boiling in Laemmli buffer for 3 minutes and were analyzed by SDS-PAGE. Detection of “bound-VDR” was done after SDS-PAGE by Western blots with VDR antibodies (Affinity BioReagents, Golden CO).

**Determination of Systemic Toxicity (Calcemia) of 25-OH-D₃-3-BE in CD-1 Mice.** Three doses of 25-OH-D₃-3-BE (3.3, 33, or 166.7 μg/kg) and two doses (3.3 or 33 μg/kg) of 25-OH-D₃ were prepared in 0.2 mL of saline-EtOH (0.1%) by diluting ethanolic solutions of the steroids with saline in such a way that the concentration of EtOH was 0.1% in the solution. These samples or saline-EtOH (0.1%) vehicle control (0.2 mL) were administered to the animals (in groups of five) intra-peri-
tonally over a period of 12 days (injection on alternate days). At the end of the experiment the animals were lightly anesthetized, and blood was collected after decapitation for serum calcium-analysis. Body weights at the beginning and at the end of the experiment were recorded.

Data Analysis. Majority of the assays was carried out in three to six replicates. Statistical analyses of the data were done with linear regression analysis and one-way ANOVA followed by Fisher’s protected least significant difference tests. P ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The 1,25(OH)2D3 and many of its synthetic analogs inhibit the growth of malignant cells (16). However, translation of the cellular results into in vivo studies has been problematic because of acute toxicity of the hormone and some of its analogs. Therefore, a major effort has been underway in designing analogs that would either inhibit cellular growth at physiologic concentrations to avoid systemic toxicity or be nontoxic at supraphysiologic doses.

VDR-binding affinity is crucial in developing 1,25(OH)2D3 analogs because of the recognition that interaction between VDR and the analogs is pivotal in the genomic process (3, 4). Therefore, analogs with relatively low VDR-binding affinity have not been considered to be of therapeutic importance. For example, 25-OH-D3, the metabolic precursor of 1,25(OH)2D3, has a poor VDR-binding affinity [Kd = 10^-6 to 10^-7 mol/L versus Kd = 10^-9 to 10^-10 mol/L for 1,25(OH)2D3]. Therefore, 25-OH-D3 and its derivatives have not been studied significantly as candidates for drug-development.

We hypothesized that covalent linking of 25-OH-D3 (via its derivative/analog) to the hormone-binding pocket of VDR might permanently lock VDR into its biologically active holo-form. This way, biologically inactive 25-OH-D3 might acquire significant cell regulatory property. Furthermore, because of the recognition that calcemic property could be separated from cell regulatory properties, the nontoxic property of 25-OH-D3 might be translated into its derivative. As a result, even supraphysiologic doses of this 25-OH-D3 analog might be used to achieve inhibition of cell growth without systemic toxicity in an in vivo system.

In a recent, study we showed that 1α,25-dihydroxyvitamin D3-3B-(2)-bromoacetate [1,25(OH)2D3-3-BE], an affinity labeling derivative of 1,25(OH)2D3, displayed strong antiproliferative effect in several normal and malignant cell lines with strongest activity toward prostate cancer cells (17–21). In the current study, we focused on a structural 25-OH-D3-prototype of 1,25(OH)2D3-3-BE (i.e., 25-OH-D3-3-BE).

Growth-inhibitory effect of 1,25(OH)2D3 and its analogs is known to vary among cell lines and even among lines from the same tissue. But, in general, strongest and predictable effect is observed at a 10^-6 mol/L concentration of the hormone or its analogs (22). Although this concentration is considered to be physiologically irrelevant, it produces optimal effect. We treated primary culture of normal human skin cells, and several cell lines including LNCaP human androgen-sensitive and PC-3 human androgen-refractory prostate cancer cells, and PZ-HPV-7 immortalized normal human prostate cells with 10^-6 mol/L of 1,25(OH)2D3 or 25-OH-D3-3-BE to compare the antiproliferative property of the analog (25-OH-D3-3-BE) with the hormone.

![Fig. 1](https://clincancerres.aacrjournals.org) [3H]Thymidine incorporation assays of keratinocytes, PC-3, LNCaP, and PZ-HPV-7 cells. Cells, grown to 60 to 70% confluence were serum starved for 20 hours followed by treatment with 10^-6 mol/L of 25-OH-D3-3-BE, 1,25(OH)2D3, 25-OH-D3, or EtOH (control) for 16 hours followed by incubation with [3H]thymidine and assaying for the incorporation of radioactivity in the cells. Results are expressed relative to EtOH control (100%). *, P < 0.00032; **, P < 0.0075. Bars, ±SD.
Effect of various agents on the growth of normal and malignant cells is often determined by [3H]thymidine incorporation assay. In this assay, increase or decrease in the incorporation of [3H]thymidine in the DNA of the growing cells by a reagent is used as an index of its proliferative/antiproliferative effect. As shown in Fig. 1, A–E, 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE and 1,25(OH)_2D_3 inhibited the growth of all the cells with various efficiency. However, the effect of 25-OH-D_3-3-BE was strongest in LNCaP and PC-3 prostate cancer cells. For example, growth of LNCaP cells were inhibited by \sim 60\% and 98\% with 1,25(OH)_2D_3 and 25-OH-D_3-3-BE, respectively (Fig. 1C), whereas growth of PC-3 cells were retarded by 70\% and 90\% by 1,25(OH)_2D_3 and 25-OH-D_3-3-BE, respectively (Fig. 1D). In contrast, growth of normal immortalized prostate cells (PZ-HPV-7 cells) were inhibited by \sim 50\% and 65\% by 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE and 10^{-6} \text{mol/L} of 1,25(OH)_2D_3, respectively (Fig. 1B). Growth inhibition by 25-OH-D_3-3-BE was stronger than an equivalent amount of 1,25(OH)_2D_3 in keratinocytes (Fig. 1A). Furthermore, 10^{-6} \text{mol/L} of 25-OH-D_3 showed marginal antiproliferative effect in PC-3 cells (Fig. 1E).

We also observed that 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE was cytotoxic only to LNCaP and PC-3 cells, causing the cells to lift, float, and die under phase contrast microscope.

In a cell counting assay, we observed that LNCaP and LAPC-4 cells had sharply reduced number with 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE after 24 hours incubation (Fig. 2A), whereas MC3T3 cells were affected to a much lesser extent, and MCF-7 cells (incubated for 48 hours) were not significantly affected. It should be noted that in this assay cells were not serum starved before addition of the reagents, and 10^{-7} \text{mol/L} of 1,25(OH)_2D_3 had little effect in all of the cells. The 10^{-7} \text{mol/L} of 1,25(OH)_2D_3 was shown to produce significant effect in LNCaP cells after a longer period (3 to 6 days) of incubation (23). However, we observed that the effect of 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE was relatively rapid (optimal antiproliferation and cytotoxicity in prostate cancer cells was observed within 16 to 24 hours of incubation). Therefore, within the short timeframe of our studies, 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE produced a strong effect in LNCaP and LAPC-4 cells, whereas 10^{-7} \text{mol/L} of 1,25(OH)_2D_3 showed very little effect, if any, in all of the cells tested.

We conducted a dose-response study in which PC-3 cells were treated with 10^{-7} \text{mol/L} and 10^{-6} \text{mol/L} of either 25-OH-D_3-3-BE or 1,25(OH)_2D_3 for 18 hours followed by [3H]thymidine incorporation assay. Results of this assay showed that 10^{-6} \text{mol/L} of 25-OH-D_3-3-BE decreased the proliferation of the cells...
by ~90%, whereas there was ~45% reduction with 10^{-6} \text{ mol/L} of 1,25(\text{OH})_2\text{D}_3. However, there was virtually no effect with 10^{-7} \text{ mol/L} of either reagent (Fig. 2B). Furthermore, we observed that 25-OH-D_3-3-BE was toxic to these cells (as well as to LNCaP cells; Fig. 1C), as they were found detached and floating.

To elaborate on the cytotoxic nature of 25-OH-D_3-3-BE, we carried out MTS cell viability assay with LNCaP, PC-3, and DU-145 cells treated with 10^{-6} \text{ mol/L} and 10^{-7} \text{ mol/L} of 25-OH-D_3-3-BE or 1,25(\text{OH})_2\text{D}_3. Results of this assay (Fig. 2C) showed that 10^{-6} \text{ mol/L} of 25-OH-D_3-3-BE reduced the number of viable cells to ~8% in LNCaP and PC-3 cells and 20% in DU-145 cells, whereas majority of the cells were viable when treated with 10^{-6} \text{ mol/L} of 1,25(\text{OH})_2\text{D}_3. With 10^{-7} \text{ mol/L} of 25-OH-D_3-3-BE and 10^{-7} \text{ mol/L} and 10^{-6} \text{ mol/L} of 1,25(\text{OH})_2\text{D}_3, majority of the cells were viable. These results suggested that 25-OH-D_3-3-BE induced toxicity in these cells at 10^{-6} \text{ mol/L}. As mentioned earlier, repeated dosing of LNCaP cells with 10^{-7} \text{ mol/L} of 1,25(\text{OH})_2\text{D}_3 for a prolonged period (48 hours) produced significant antiproliferative effect, whereas a single dose and shorter incubation period failed to produce such an effect (23). Therefore, it is probable that repeated dosing with 10^{-7} \text{ mol/L} of 25-OH-D_3-3-BE for longer periods (we typically dosed the cells for 16 to 20 hours) might have produced significant antiproliferative and possibly cytotoxic effects.

Induction of toxicity in DU-145 cells deserves special attention, because it has been shown that DU-145 cells respond poorly to 1,25(\text{OH})_2\text{D}_3-treatment because of enhanced activity of the catabolic enzyme, 24-OHase (24, 25). We postulated that covalent attachment of 25-OH-D_3-3-BE into the ligand-binding pocket of VDR might prevent the catabolism of the analog and produce sufficient quantity of transcriptionally active VDR. Therefore, our results with DU-145 cells lend strong support for this hypothesis.

The growth inhibitory effect of 1,25(\text{OH})_2\text{D}_3 and its analogs is generally manifested via the arresting of cellular growth in G_0/G_1 phase; and such activity correlates well with the expression of cyclin-dependent kinase inhibitors, such as p21 and p27 (26). However, in some cases, apoptosis, or programmed cell death, has been reported. For example, it was reported that 1,25(\text{OH})_2\text{D}_3 induced apoptosis in MCF-7 cells (22, 27), although in prostate cancer cells reports are conflicting. For example, Blutt et al. (23) reported that 1,25(\text{OH})_2\text{D}_3 induced apoptosis in LNCaP cells, but another group failed to observe such an effect (28).

Fragmentation of nuclear DNA is a hallmark of the downstream process manifested by cells undergoing apoptosis. When PC-3 cells were treated with 0.25 \times 10^{-6} \text{ mol/L} of 25-OH-D_3-3-BE, 25-OH-D_3, or 1,25(\text{OH})_2\text{D}_3, DNA-fragmentation was observed only with cells treated with 25-OH-D_3-3-BE (Fig. 3A, Lane 4), whereas no such effect was visible with an equivalent amount of 1,25(\text{OH})_2\text{D}_3 (Fig. 3A, Lane 2) or 25-OH-D_3 (Fig. 3A, Lane 3). These results suggested that 25-OH-D_3-3-BE induced apoptosis in PC-3 cells, whereas an equivalent amount of 25-OH-D_3 and 1,25(\text{OH})_2\text{D}_3 failed to do so.

Caspases are key indicators of apoptosis in cells (29). 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, and so forth. 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 procaspase 3, which in turn is manifested through classical features of apoptosis such as cleavages of PARP, U1-ribonucleoprotein, and so forth.

Recently, Guzey et al. (30) reported that 1,25(OH)_2D_3 activated caspase 3 and caspase 9, but not caspase 8, in ALVA-31 cells. Polek et al. (31) also showed that 1,25(OH)_2D_3 did not induce apoptosis in PC-3 cells. When PC-3 cells were treated with 0.01 \times 10^{-6} \text{ mol/L} of 25-OH-D_3-3-BE or 25-OH-D_3 or 1,25(OH)_2D_3, only 25-OH-D_3-3-BE showed strong induction of caspases 3, 8, and 9 (Fig. 3B). Therefore, DNA-fragmentation analysis and caspase-activation assay collectively suggested that 25-OH-D_3-3-BE induced apoptosis in PC-3 cells.

The 25-OH-D_3-3-BE contains an ester bond. Therefore, esterases in growing cells might hydrolyze this molecule to

![Figure 3: DNA Fragmentation Analysis](image-url)
produce equimolar amounts of 25-OH-D₃ and bromoacetic acid (Fig. 4, top panel). It could be argued that the observed effects of 25-OH-D₃-3-BE might be because of bromoacetic acid, 25-OH-D₃, or a combination of the two. To determine any role of in situ-produced bromoacetic acid (by the hydrolysis of 25-OH-D₃-3-BE), we carried out [³H]thymidine incorporation assay in PC-3 cells treated with 10⁻⁶ mol/L of either bromoacetic acid or 25-OH-D₃-3-BE or a mixture containing 10⁻⁶ mol/L each of bromoacetic acid or 25-OH-D₃-3-BE. As shown in Fig. 4 (bottom left panel), 10⁻⁶ mol/L of 25-OH-D₃-3-BE was strongly antiproliferative to the cells, whereas 10⁻⁶ mol/L of bromoacetic acid did not have any significant effect on the proliferation of these cells. Furthermore, a mixture containing 10⁻⁶ mol/L each of bromoacetic acid and 25-OH-D₃-3-BE produced the same effects as 10⁻⁶ mol/L of 25-OH-D₃-3-BE alone (Fig. 4, bottom right panel). Therefore, these results strongly suggested that the observed properties of 25-OH-D₃-3-BE were related to its unhydrolyzed (intact) form.

However, the above results did not rule out the possibility that a part of 25-OH-D₃-3-BE might undergo hydrolysis, and 25-OH-D₃, produced in situ by this hydrolytic process, might be metabolically activated by 25-hydroxyvitamin D₃-1α-hydroxylase (1-OHase) to 1,25(OH)₂D₃, which could in turn produce the observed effects, at least partially. LNCaP cells are known to be deficient in the 1-OHase enzyme (32), yet 25-OH-D₃-3-BE showed strong antiproliferative effect in these cells (Fig. 1 C). These considerations essentially ruled out any role of in situ-produced 25-OH-D₃ in the observed antiproliferative and cytotoxic properties of 25-OH-D₃-3-BE.

The 25-OH-D₃-3-BE contains a chemically reactive α-halocarbonyl group; therefore, it could potentially alkylate any protein in a cellular system, and such random interaction could possibly be responsible for its observed effects. FBS contains many proteins, including a relatively large amount of vitamin D-binding protein, which could potentially react with 25-OH-D₃-3-BE, and eliminate it completely before it reacts with VDR. Typically, the assays described here were carried out in a media containing 5 to 10% FBS, suggesting that scavenging of 25-OH-D₃-3-BE by serum vitamin D-binding protein (and other cellular proteins in a random fashion) might not play a significant role in the observed properties of this compound.

Because VDR was our desired target to elicit the biological activity of 25-OH-D₃-3-BE, it became obligatory for us to show the involvement of processes related to 1,25(OH)₂D₃/VDR-signaling pathways. The 24-OHase gene is known to be strongly and predictably modulated by 1,25(OH)₂D₃ and its analogs. We carried out a study to evaluate the effect of 1,25(OH)₂D₃ and 25-OH-D₃-3-BE at various doses on the 24-OHase promoter activity in COS-7 cells that was transfected with a VDR construct, tagged with a CAT reporter gene. Results of this assay, shown in Fig. 5, showed that 24-OHase promoter activity was strongly up-regulated by 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L of 1,25(OH)₂D₃. In contrast, strong promoter activity was displayed only with 10⁻⁶ mol/L of 25-OH-D₃-3-BE, and such activity declined almost to the basal level with 10⁻⁷ mol/L of 25-OH-D₃-3-BE. These results strongly suggested that the molecular action of 25-OH-D₃-3-BE might follow a path similar to 1,25(OH)₂D₃, however, with less efficiency.

An important aspect of ligand-receptor interaction is the ability of the hormone and the analogs to induce transcriptionally active conformation in VDR that can interact with RXR and other coactivators required for transcription, such as GRIP-1 (33). Therefore, to determine the potency of 1,25(OH)₂D₃ and 25-OH-D₃-3-BE to induce interaction of VDR with RXR and/or with the steroid receptor coactivator, GRIP-1, we used a pull
down assay in which PC-3 cells were incubated with various doses of 1,25(OH)2D3 or 25-OH-D3-3-BE, and VDR-interacting proteins were pulled down with GST-fused GRIP or RXR. Results of these assays showed strong interaction between VDR and GRIP-1 when the cells were incubated for 1 hour with 25-OH-D3-3-BE (10^{-6} mol/L) or 1,25(OH)2D3 (10^{-7} mol/L; Fig. 6, left panel). However, after 24 hours of incubation, strong interaction between VDR and GRIP-1 was observed with 10^{-7} mol/L of 25-OH-D3-3-BE. With RXR, there was significant interaction even with 10^{-8} mol/L of 25-OH-D3-3-BE (Fig. 6, right panel).

The above results provided the evidence that 25-OH-D3-3-BE was able to activate VDR at substantially lower concentrations in PC-3 cells; which is consistent with the results of DNA-fragmentation and caspase-activation analysis. However, a significantly higher dose (10^{-6} mol/L) of 25-OH-D3-3-BE was required to show 24-OHase–promoter activity in COS cells as well as antiproliferative activity in various cells. These discrepancies underscore the hypothesis that gene regulatory events leading to inhibition of cell growth might be different from those leading to apoptosis. Whether or not all of these cellular events are mediated through transcriptional activity of the VDR remains to be established. Furthermore, differences in the potency of analogs to induce different gene regulatory events through VDR in the same cell type have been reported by several studies, including Shevde et al. (34). This study with 2MD, an analog of 1,25(OH)2D3, showed a range of sensitivity for regulating gene expression, from ED50 = 10^{-11} mol/L for the up-regulation of RANKL to ED50 > 10^{-10} mol/L for induction of the VDR responsive genes, osteopontin and 24-hydroxylase in mouse osteoblasts. Likewise, Ismail et al. (35) showed that the analog Ro-26-9228 had an ED50 of 2.1 \times 10^{-8} mol/L for the induction of 24-OHase and an ED50 of 2.65 \times 10^{-7} mol/L for induction of Calbindin D9k in Caco-2 cells.

A major concern involving 1,25(OH)2D3 and its analogs is systemic toxicity (hypercalcemia, hypercalciuria) that is often found to be associated with these molecules. Therefore, if 25-OH-D3-3-BE and related compounds were to be developed as therapeutic agents, they should be devoid of systemic toxicity. Although it is difficult to draw a direct correlation between in vitro and in vivo dosages, it was clear that doses (of 25-OH-D3-3-BE) that might be required to reach a potential therapeutic level would be significantly higher than what has been customary.

**Fig. 5** Analysis of 24-OHase promoter activity in COS-7 cells, transiently transfected with a 24-OHase-construct, tagged with a chloramphenicon (CAT) reporter gene, and hVDR expression vector. Cells were treated with various doses (as indicated) of 25-OH-D3-3-BE or 1,25(OH)2D3, and CAT activity was determined as described in Materials and Methods. % Maximal in the X-axis denotes percentage of maximum activity (in this case with 10^{-6} mol/L of 25-OH-D3-3-BE). *, P < 0.005; **, P < 0.0001. Bars, \pm SD.

**Fig. 6** Pull down assays in PC-3 cells to determine the interaction between VDR and RXR and GRIP-1, as the cells were treated with various doses of 25-OH-D3-3-BE or 1,25(OH)2D3, followed by incubation with GST-RXR or GST-GRIP-1 fusion proteins. The RXR and GRIP-1 bound proteins were adsorbed on glutathione-Sepharose beads. The bound proteins were eluted from the beads by boiling in Laemmli buffer and were analyzed by polyacrylamide gel electrophoresis. The protein bands were transferred onto a polyvinylidene difluoride membrane, and blots were visualized by Western blotting with an anti-VDR antibody.
with 1,25(OH)₂D₃ and its analogs. However, we surmised that an analog of 25-OH-D₃/1,25(OH)₂D₃ could be useful in higher concentrations as long as it did not show systemic toxicity. For example, higher than customary doses of 1α-hydroxyvitamin D₃ were used *in vivo* to elicit desired effects (36).

We carried out a toxicity study of 25-OH-D₃-BE in CD-1 mice where we used 25-OH-D₃ as a control. Our purpose was to determine whether we could extrapolate the nontoxic property of 25-OH-D₃ to its analog (*i.e.*, 25-OH-D₃-BE) and to obtain a preliminary idea about the safe dose levels of 25-OH-D₃-BE. As shown by the results (Table 1), there was no significant difference in serum calcium values and weights of the animals from the vehicle control with 3.3 or 33 μg/kg of 25-OH-D₃ or 25-OH-D₃-BE. Although there was a slight increase in serum calcium value only with the highest dose (166.7 μg/kg) of 25-OH-D₃-BE, body weights of the animals were not significantly different from the vehicle control. It should be emphasized that the above results simply denoted that 25-OH-D₃-BE had a significantly lower toxicity than 1,25(OH)₂D₃ or majority of its analogs without providing any information on its effective serum concentration and bioavailability. We have shown that 25-OH-D₃-BE is the active molecule that is responsible for the observed antiproliferative activity in prostate cancer cells (Fig. 4). But, we appreciate that 25-OH-D₃-BE can undergo hydrolytic cleavage *in vivo* to reduce its bioavailability. In the future, we will carry out pharmacokinetic and pharmacodynamic studies to shed light on this issue.

In toxicity studies, it is customary to use 1,25(OH)₂D₃ as a control. But 1,25(OH)₂D₃ and many of its synthetic analogs are known to be toxic at doses used in our study. For example, in a recent publication it was reported that 1.0 μg/kg of 1,25(OH)₂D₃ and EB-1089 [a noncalcemic analog of 1,25(OH)₂D₃] raised serum calcium beyond vehicle control, although significantly less with EB-1089 than 1,25(OH)₂D₃ (37). For obvious reasons, we could not use 1,25(OH)₂D₃ or EB-1089 as controls at high dose levels that were used in our toxicity study with 25-OH-D₃-BE.

The 1,25(OH)₂D₃ and its analogs are generally not known to have tissue/tumor specific effects because of the ubiquitous nature of VDR, the chief modulator of their actions. In this communication, we report that 25-OH-D₃-BE, a VDR-affinity alkylating derivative of the prehormone, displayed strong antiproliferative activity in keratinocytes was much lower than 1,25(OH)₂D₃-3-BE. This suggested that covalent modification of a specific area of VDR [by 3-boronoacetates: 25-OH-D₃-3-BE and 1,25(OH)₂D₃-3-BE] has a profound effect on transcriptional activities. We postulate that 25-OH-D₃-3-BE changes the conformation of VDR (on alkyla
tion) so that the liganded receptor specifically and uniquely modulate certain factor/factors directly or indirectly in the prostate cancer cells. We are currently in the process of identifying such factor/factors by gene-profiling experiments.

Several clinical trials involving 1,25(OH)₂D₃ and its analogs in prostate and other cancers are currently underway. Results of the studies described in this report strongly suggest that 25-OH-D₃-3-BE and related VDR-cross linking analogs of 25-OH-D₃ might be useful as potential therapeutic agents for androgen-sensitive and androgen-refractory prostate cancer.

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**REFERENCES**

Inhibition of Proliferation and Induction of Apoptosis by 25-Hydroxyvitamin D$_3$-$3\beta$-(2)-Bromoacetate, a Nontoxic and Vitamin D Receptor-Alkylation Analog of 25-Hydroxyvitamin D$_3$ in Prostate Cancer Cells

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