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Mechanistic and pharmacodynamic studies of a 25-hydroxyvitamin D_3 derivative in prostate cancer cells

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Abstract

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 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 D_3-3-bromoacetate (25-OH-D_3-3-BE), a derivative of 25-hydroxyvitamin D_3, the pro-hormonal form of $1,25(OH)_2D_3$ 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-D_3-3-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-D_3-3-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-D_3-3-BE to demonstrate potential therapeutic applicability of 25-OH-D_3-3-BE in hormone-sensitive and hormone-insensitive prostate cancer.

Keywords: 25-Hydroxyvitamin D_3-derivative; 1,25-Dihydroxyvitamin D_3; 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 receptor 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 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 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 $1,25(OH)_2D_3$ because this dihydroxy metabolite of vitamin D_3 is biologically the most active form of the hormone. Although 25-hydroxyvitamin

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D_3 (25-OH-D_3), the non-toxic pre-hormonal form of 1,25$(OH)_2D_3$ 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-D_3 as a potential antiproliferative agent for prostate cancer therapy [16,17]. We reported that 25-hydroxyvitamin D_3-3β-(2)-bromoacetate (25-OH-D_3-3-BE), a derivative of 25-OH-D_3, 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-D_3-3-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-D_3-3-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-D_3-3-BE and 25-hydroxyvitamin D_3-3β-[2^{14}C]-bromoacetate [^{14}C-25-OH-D_3-3-BE] (sp. activity 14.3 mCi/mmol) was synthesized according to published procedures from our laboratory [19]. [25(26)-^{3}H]25-Hydroxyvitamin D_3-3β-bromoacetate [^{3}H-25-OH-D_3-3-BE] (sp. activity 0.02 μCi/μmol) was synthesized by spiking a sample of 25-OH-D_3 with [25(26)-^{3}H]25-hydroxyvitamin D_3 (50,000 cpm, specific activity 20.6 Ci/mmol and treating the mixture with bromoacetaic 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$(OH)_2D_3$ or 25-OH-D_3-3-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$(OH)_2D_3$, 25-OH-D_3-3-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$(OH)_2D_3$ or 25-OH-D_3-3-BE or ethanol control and allowed to incubate 24 h in a humidified 37 °C, 5% CO_2 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 Na_3VO_4) containing Roche Complete Protease Inhibitors. Cell lysates were subjected to centrifugation (13,000*g*, 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 ^{14}C-25-OH-D_3-3-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 ^{14}C-25-OH-D_3-3-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% H_2O–MeOH) for HPLC analysis. These extracts were analyzed by reverse phase HPLC using an Agilent 5 μm C_{18} column, 10% H_2O 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-D_3-3-BE was analyzed by the same system. This assay was run in duplicate.

Serum-stability of ^{3}H-25-OH-D_3-3-BE. A 0.5 ml aliquot of a pooled human serum sample was incubated with ^{3}H-25-OH-D_3-3-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% H_2O 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-D_3 and 25-OH-D_3-3-BE was run in the HPLC as a standard.

Results and discussion

The antiproliferative effect of 25-OH-D_3-3-BE is mediated by VDR in ALVA-31 prostate cancer cells

In previous studies, we described that 25-OH-D_3-3-BE, a derivative of 25-OH-D_3 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-D_3-3-BE induces 1α,25-dihydroxyvitamin D_3-24-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-D_3-3-BE are similar to those of 1,25$(OH)_2D_3$, and mediated by a VDR-activation pathway.

To confirm that the growth inhibitory properties of 25-OH-D_3-3-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$(OH)_2D_3$ is manifested via its interaction with VDR in ALVA-31 cells [20], if the antiproliferative effects of 25-OH-D_3-3-BE is also modulated through VDR, we can expect that 25-OH-D_3-3-BE-mediated

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

As shown in Fig. 1 growth of VDR-sense cells (empty vector) is strongly inhibited by $10^{-7\text{–}6}$ M of $1,25(OH)_2D_3$ as reported earlier [20]. Conversely, the growth of antisense cells treated with $10^{-7\text{–}6}$ M of $1,25(OH)_2D_3$ is similar to that of ethanol-control, confirming the requirement of VDR in the antiproliferative activity of $1,25(OH)_2D_3$ in ALVA-31 cells. In the case of 25-OH-D_3-3-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-D_3-3-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-D_3-3-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-D_3-3-BE in prostate cancer cells.

We observed that 25-OH-D_3-3-BE is approximately one log scale less efficient than $1,25(OH)_2D_3$ in inhibiting the growth of wild type ALVA-31. Earlier we reported similar dose-dependence (of 25-OH-D_3-3-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 $1,25(OH)_2D_3$ shows a range of sensitivity for regulating gene expression from $ED_{50} = 10^{-11}$ mol/L for the up-regulation of RANKL to $ED_{50} > 10^{-10}$ mol/L for induction of osteopontin and 24-OHase in mouse osteoblasts [23]. Similarly, it was shown that RO-26-9228, an analog of $1,25(OH)_2D_3$ 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-D_3-3-BE compared with $1,25(OH)_2D_3$ in modulating gene-regulatory events is not unexpected.

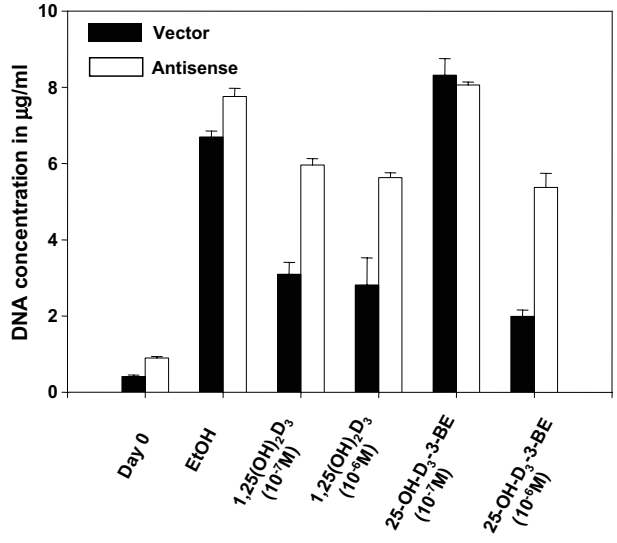

Fig. 1. 25-OH-D_3-3-BE inhibits the growth of ALVA-31 prostate cancer cells through a VDR-dependent mechanism. ALVA-31 control (vector) and ALVA-31 VDR "null" cells (antisense) were treated with the indicated doses of $1,25(OH)_2D_3$, 25-OH-D_3-3-BE and ethanol control for 6 days. Monolayers were harvested for DNA quantification. Each condition was conducted in triplicate. Values are presented as mean DNA concentration ± standard error.

25-OH-D_3-3-BE inhibits Akt phosphorylation in PC3 prostate cancer cells

Previously, we reported that 25-OH-D_3-3-BE induced nuclear DNA-fragmentation and activated caspases 3, 8 and 9, hallmarks of apoptosis, in PC3 cells while an equimolar concentration of $1,25(OH)_2D_3$ and 25-OH-D_3 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-D_3-3-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-D_3-3-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-D_3-3-BE, while Akt phosphorylation was unaffected by $1,25(OH)_2D_3$.

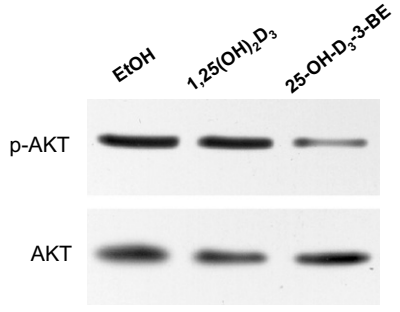

Fig. 2. 25-OH-D_3-3-BE inhibits Akt phosphorylation in PC-3 prostate cancer cells. PC-3 cells were treated with equimolar concentrations of $1,25(OH)_2D_3$, 25-OH-D_3-3-BE and ethanol control and Western analysis performed for phosphorylated Akt (p-Akt). The blot was stripped and re-probed for total Akt to ensure equal loading of protein in the lanes.

These results suggested that 25-OH-D_3-3-BE may exert its antiproliferative effects, at least in part, by inhibiting this pro-survival pathway.

25-OH-D_3-3-BE is taken up in its intact form by DU-145 cells

The antiproliferative and apoptotic activities of 25-OH-D_3-3-BE in prostate cancer cells strongly endorse its potential 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-D_3-3-BE contains a hydrolytically unstable ester bond and its hydrolysis would produce equivalent amounts of 25-OH-D_3 and bromoacetic acid. In an earlier study we demonstrated that the growth inhibitory property of 25-OH-D_3-3-BE is related strictly to the intact molecule

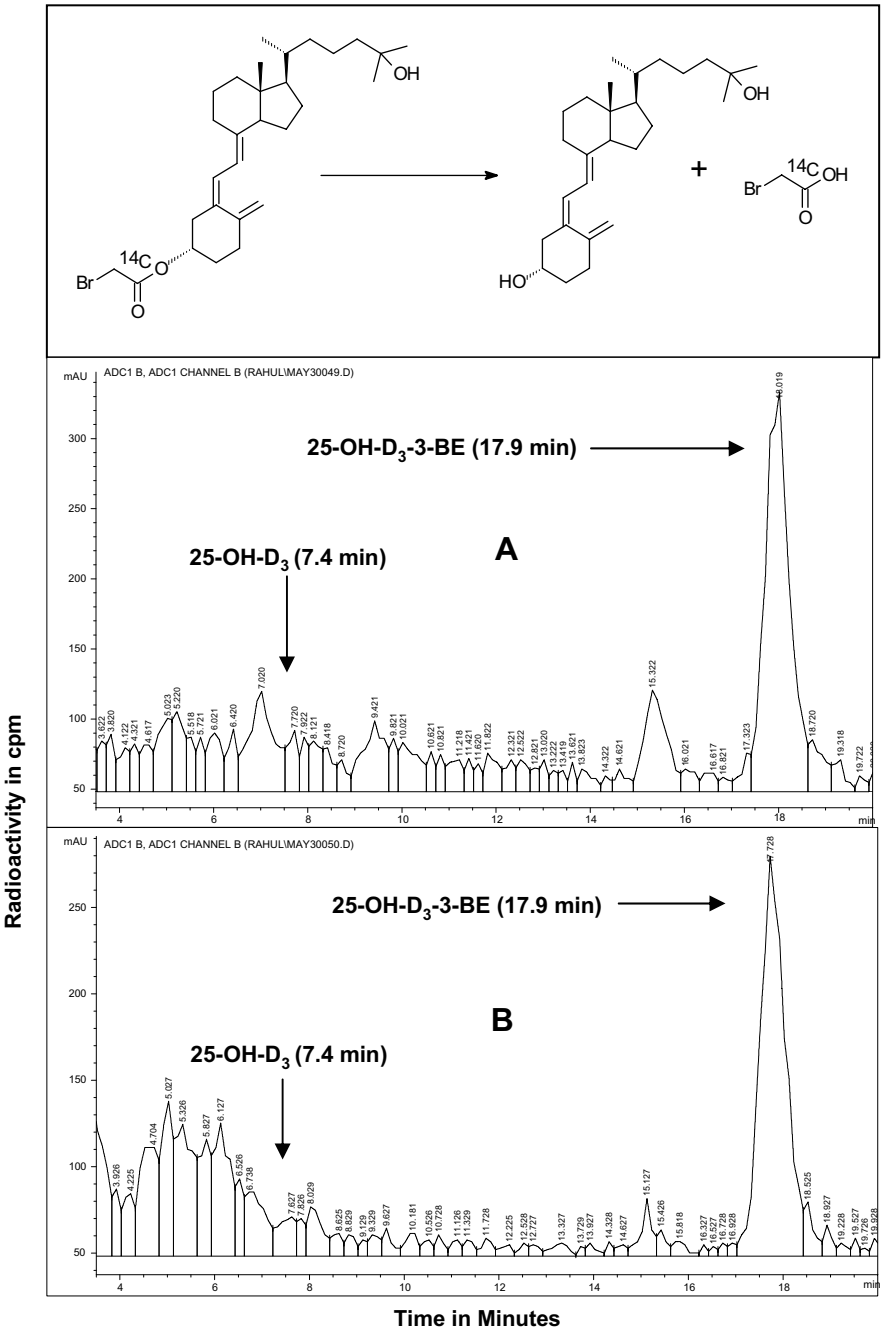

Fig. 3. 25-OH-D_3-3-BE is taken up by DU-145 prostate cancer cells in its intact form. DU-145 prostate cancer cells were treated with ^{14}C-25-OH-D_3-3-BE and HPLC analysis performed on the media (A) and whole cell extracts (B). Fractions were counted for radioactive content. Hydrolysis of ^{14}C-25-OH-D_3-3-BE producing unlabeled 25-OH-D_3 and ^{14}C-bromoacetic acid is shown in the top of the figure. (A) Media extract; (B) Cellular extract. Position of the 25-OH-D_3 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-D_3-3-BE in DU-145 cells. The goal of this study was to determine whether we can isolate 25-OH-D_3-3-BE in its intact form from cellular extracts. For this study we employed a radiolabeled version of 25-OH-D_3-3-BE, i.e. 25-hydroxyvitamin D_3-3β-[2^{14}C]-bromoacetate (^{14}C-25-OH-D_3-3-BE). We argued that hydrolysis of ^{14}C-25-OH-D_3-3-BE should produce unlabeled 25-OH-D_3 and radiolabeled bromoacetic acid (^{14}C-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-D_3-3-BE would represent intact 25-OH-D_3-3-BE.

HPLC analysis of the organic extracts of media and DU-145 cells incubated with ^{14}C-25-OH-D_3-3-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 ^{14}C-25-OH-D_3-3-BE (Fig. 3, middle 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-D_3-3-BE is taken up by the cells in its intact form.

25-OH-D_3-3-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-D_3-3-BE and then carry out an organic solvent extraction and HPLC-analysis of the extract. We argued that ^{14}C-25-OH-D_3-3-BE, used for the previous study, could not be used here, because hydrolysis of ^{14}C-25-OH-D_3-3-BE would result in a loss of radioactivity (as ^{14}C-bromoacetic

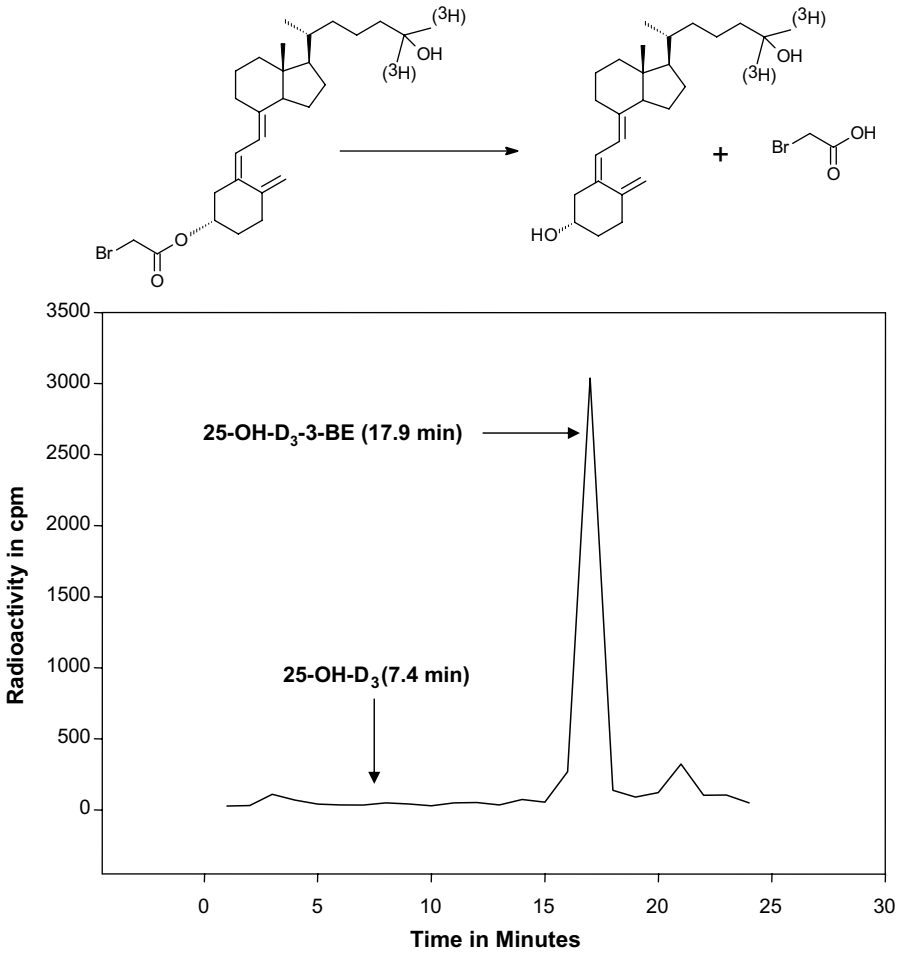

Fig. 4. 25-OH-D_3-3-BE is stable in human serum. ^{3}H-25-OH-D_3-3-BE was synthesized and incubated with human serum, followed by extraction with an organic solvent, and HPLC analysis of the organic extract. Fractions from HPLC were mixed with scintillation cocktail and counted for radioactivity. (Upper panel) Representation of the hydrolysis of ^{3}H-25-OH-D_3-3-BE leading to the production of ^{3}H-25-OH-D_3 and unlabeled bromoacetic acid. (Lower panel) Peak indicating intact ^{3}H-25-OH-D_3-3-BE. Position of the 25-OH-D_3 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-D_3-3-BE in serum we synthesized ^{3}H-25-OH-D_3-3-BE in which the radiolabel (^{3}H) is in the 25-OH-D_3 moiety. Hence, its hydrolysis would produce ^{3}H-25-OH-D_3 and unlabeled bromoacetic acid (as noted in Fig. 4, inset); and the organic extract will thus contain a combination of ^{3}H-25-OH-D_3 and ^{3}H-25-OH-D_3-3-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 ^{3}H-25-OH-D_3-3-BE. The absence of a radioactive peak corresponding to ^{3}H-25-OH-D_3 (hydrolysis product) indicates that ^{3}H-25-OH-D_3-3-BE is fully stable under these experimental conditions. Absence of hydrolysis also suggests that 25-OH-D_3-3-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-D_3-3-BE are mediated by a VDR-dependent pathway similar to the native hormone, 1,25$(OH)_2D_3$, while its apoptotic property is manifested via activation of Akt-phosphorylation pathway. Additionally, results included in this communication demonstrate favorable phramacodynamic properties, including cellular uptake in intact form and serum-stability leading to the belief that 25-OH-D_3-3-BE can potentially be developed as a therapeutic agent for prostate cancer.

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