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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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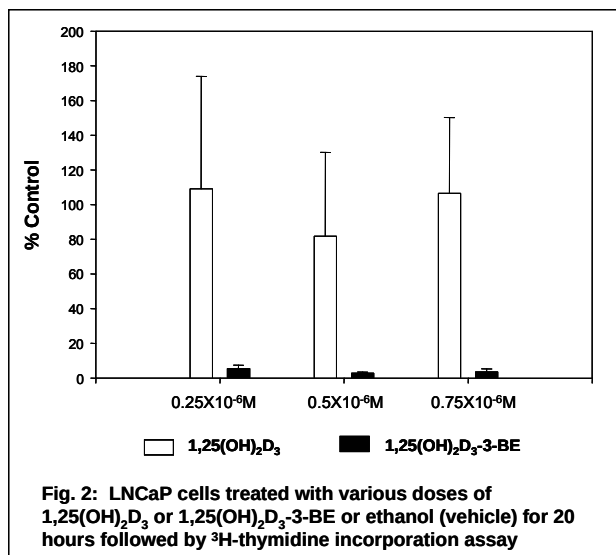
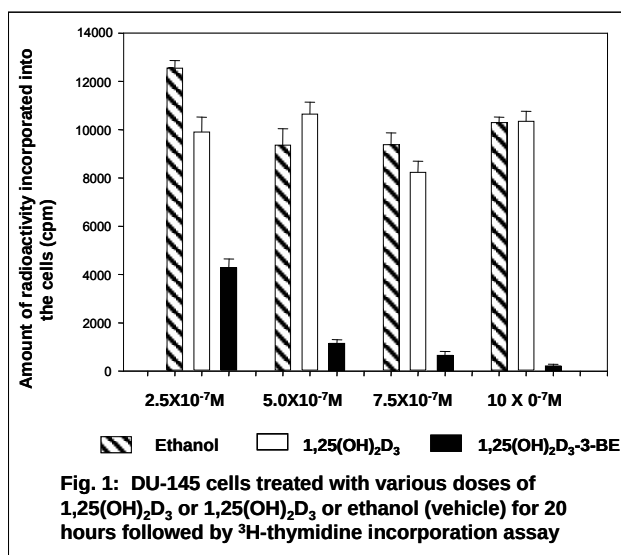
Introduction

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 3) of the project

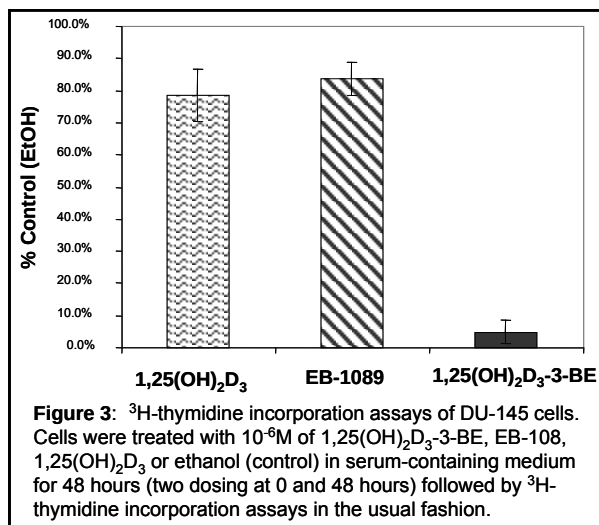
I. Continuation of studies to evaluate effects of 1,25(OH)₂D₃-3-BE in various prostate cancer cell lines

1. Dose response studies of the antiproliferative effect of 1,25(OH)₂D₃-3-BE and 1,25(OH)₂D₃ in LNCaP (androgen-sensitive PC cells) and DU-145 (androgen-insensitive PC cells):



LNCaP and DU-145 cells were grown to approximately 60% confluence in RPMI medium with 5% FBS and then treated with various doses 1,25(OH)₂D₃-3-BE and 1,25(OH)₂D₃ in serum-containing medium for 20 hours followed by ³H-thymidine incorporation assay. Results of this assay demonstrate that 1,25(OH)₂D₃-3-BE is strongly antiproliferative in a dose-dependent manner in both cell-lines. However, equivalent amounts of 1,25(OH)₂D₃ showed significantly lower or no effects in these cells (Figures 1 & 2).

2. Comparison of the effects of 1,25(OH)₂D₃-3-BE, 1,25(OH)₂D₃ and EB-1089 in DU-145 cells:



At present EB-1089 is the most promising non-calcemic analog of 1,25(OH)₂D₃ that has shown strong promise in inoperable hepatocellular carcinoma. We compared the antiproliferative effect of EB-1089, 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE in DU-145 cells by ³H-thymidine incorporation assay. As shown in **Figure 3**, only 1,25(OH)₂D₃-3-BE showed a strong antiproliferative effect in DU-145 cells.

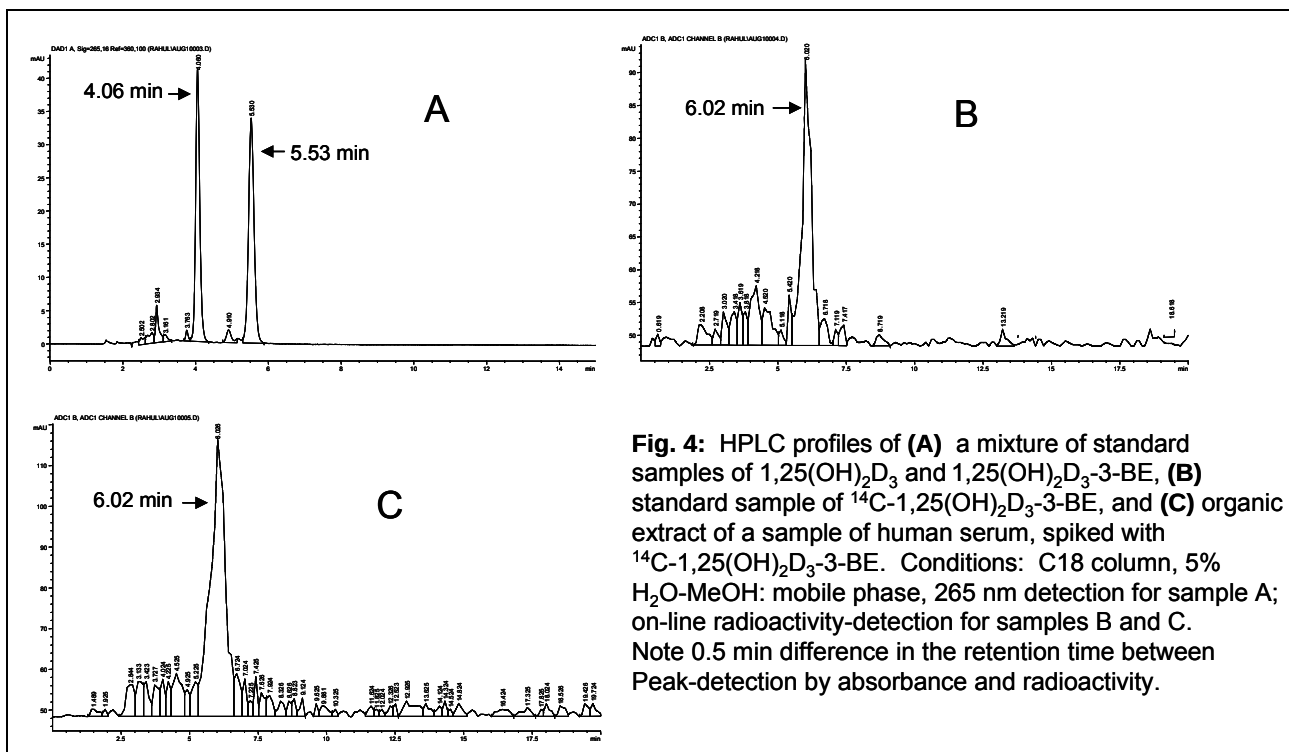
Collectively, above results showed a strong potential of 1,25(OH)₂D₃-3-BE in prostate cancer.

II. Pharmacokinetic studies of 1,25(OH)₂D₃-3-BE

1. Serum-stability of 1,25(OH)₂D₃-3-BE:

Stability of a drug in serum is one of the most important pharmacokinetic properties. Therefore, we determined the stability of 1,25(OH)₂D₃-3-BE in human serum.

Procedure: Pooled human serum (1 ml) was spiked with 1,25-dihydroxyvitamin D₃-3-bromo[1-¹⁴C]acetate (¹⁴C-1,25(OH)₂D₃-3-BE, sp. activity 14.8 mCi/mmol, 100,000 cpm) for one hr at 37°C followed by extraction with 5 x 1 ml of ethyl acetate. Combined organic extract was



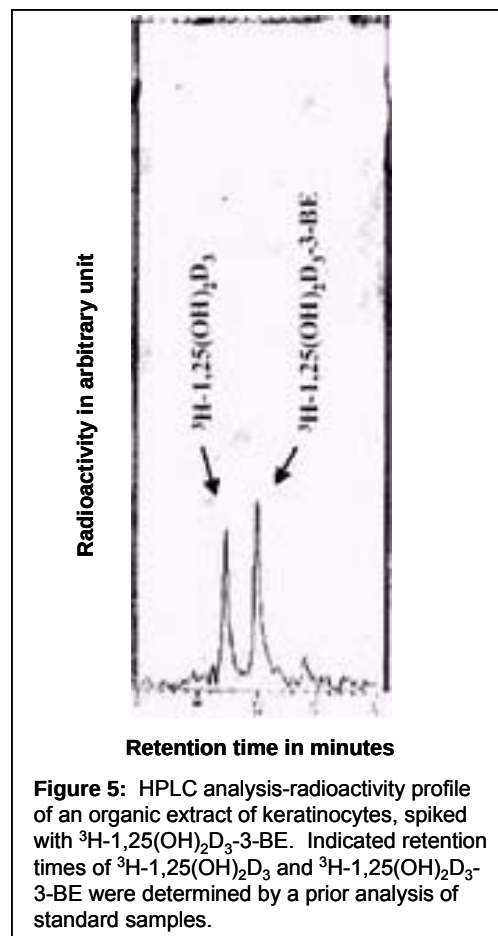
dried under argon and the residue was re-dissolved in a small volume of 5% H₂O-MeOH, and analyzed in an Agilent 1100 Series HPLC system (Thermo-Fisher, Waltham, MA), connected to a Packard Flow Scintillation Analyzer (Model no. 150TR, Meriden, CT), using 5% H₂O-MeOH as mobile phase, flow rate 1.5 ml/min, detection 265 nm (for non-radioactive materials), Agilent

C18 analytical column (Thermo-Fisher, Waltham, MA). Prior to the analysis of the radioactive sample a mixture of standard samples of $1,25(\text{OH})_2\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ -3-BE was analyzed by HPLC under the same conditions as stated before.

Results of this analysis are shown in **Figure 4**. HPLC-profile of an organic extract of the serum sample, spiked with ^{14}C - $1,25(\text{OH})_2\text{D}_3$ -3-BE showed the intact peak of ^{14}C - $1,25(\text{OH})_2\text{D}_3$ -3-BE after one hr incubation at 37°C attesting to the stability of $1,25(\text{OH})_2\text{D}_3$ -3-BE in serum.

2. Cellular uptake analysis of ^3H - $1,25(\text{OH})_2\text{D}_3$ -3-BE in keratinocytes

An important factor in the potential development of $1,25(\text{OH})_2\text{D}_3$ -3-BE as a therapeutic agent for prostate cancer is its bio-availability. $1,25(\text{OH})_2\text{D}_3$ -3-BE is an alkylating agent. Therefore, it can be scavenged by irrelevant proteins. Furthermore, it contains an esterase-labile ester bond; and therefore, it can be hydrolyzed completely before interacting with VDR, the target. Therefore, it is particularly important to determine whether this molecule is internalized by the cells in its intact form, or not. We addressed this issue by treating keratinocytes with ^3H -labeled $1,25(\text{OH})_2\text{D}_3$ -3-BE, and extracting the cells with an organic solvent followed by HPLC-analysis of the organic extract.



Procedure: Keratinocytes were grown in 35 mm plates to 60% confluence when the media was replaced with fresh media containing 50,000 cpm of ^3H -labeled $1,25(\text{OH})_2\text{D}_3$ -3-BE [$1,25$ -dihydroxy $\{25,26\text{-}^3\text{H}\}$ vitamin D_3 -3-bromoacetate, sp. activity 100 Ci/mM]. After one hour of incubation media was withdrawn and the cells were thoroughly washed with PBS. After the wash cells were lysed by adding 1 ml of PBS and 5 ml of methanol to the plates. Then the cells were scraped with a rubber policeman and transferred to a test tube. The mixture was centrifuged at 4,000 rpm and the supernatant was collected and evaporated to dryness with argon. The residue was dissolved in a small volume of 10% water in methanol and injected into a Waters HPLC system fitted with a Milipore microBondapak C_{18} column (5μ). The column was eluted at a flow rate of 1 ml/min. Effluent from the HPLC was introduced directly into a FloOne Beta Online Radioactivity Detector (Packard Instruments). Standard samples of $1,25(\text{OH})_2\text{D}_3$ -3-BE and $1,25(\text{OH})_2\text{D}_3$ were analyzed under the same conditions to determine their retention times.

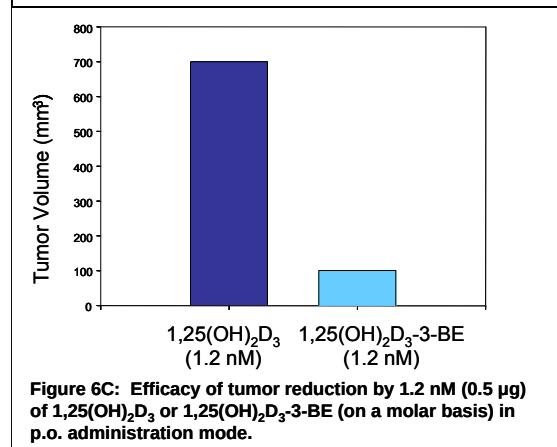
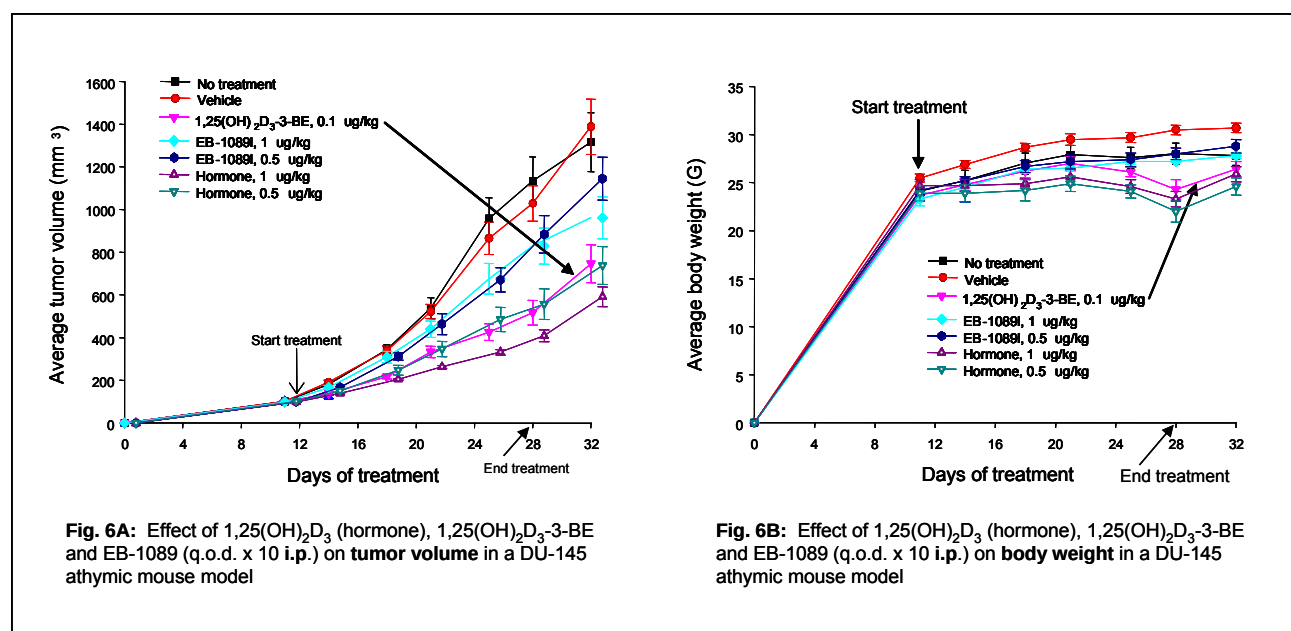
Results: The radioactivity profile of the HPLC chromatographic analysis of the cellular extract, shown in **Figure 5** demonstrate that approximately 40% of ^3H - $1,25(\text{OH})_2\text{D}_3$ -3-BE is hydrolyzed to ^3H - $1,25(\text{OH})_2\text{D}_3$, but rest of the material is present as ^3H - $1,25(\text{OH})_2\text{D}_3$ -3-BE. Therefore, this results strongly indicated that a significant portion of $1,25(\text{OH})_2\text{D}_3$ -3-BE is internalized by the cells to promote its biological activity. Therefore, we conclude that even some portion of $1,25(\text{OH})_2\text{D}_3$ -3-BE is hydrolyzed, the remainder of the intact (un-hydrolyzed) form is sufficient to bring about the observed cellular effects.

III. *In vivo* efficacy studies of 1,25(OH)₂D₃-3-BE, 1,25(OH)₂D₃ and EB-1089 in athymic mice inoculated with DU-145 human prostate cancer cells:

Male, athymic mice (Charles River Laboratories, Wilmington, MA, average weight 20 gm) were fed normal rat chow and water *ad libitum*. They were inoculated with DU 145 cells, grown in culture in the flank under light anesthesia. When the tumor size grew to approximately 100 mm³ the animals were randomized into groups of ten (10) tumor-bearing animals, and they were given 1,25(OH)₂D₃-3-BE (0.1 µg/kg), 1,25(OH)₂D₃ (0.5 and 1 µg/kg), EB-1089 (0.5 and 1 µg/kg), and vehicle (5% dimethylacetamide, DMA in sesame oil) by either intraperitoneal injection (*i.p.*) or oral gavage (*p.o.*) on approximately every third day and one group was left untreated. Treatment started on day 11 and stopped on day 28; and they were left untreated for four (4) additional days when they were sacrificed and blood samples were collected for serum calcium analysis. Body-weights of the animals were measured on the days of administration of various agents.

Results (*i.p.*-administration, Figure 6A & 6B, 6C):

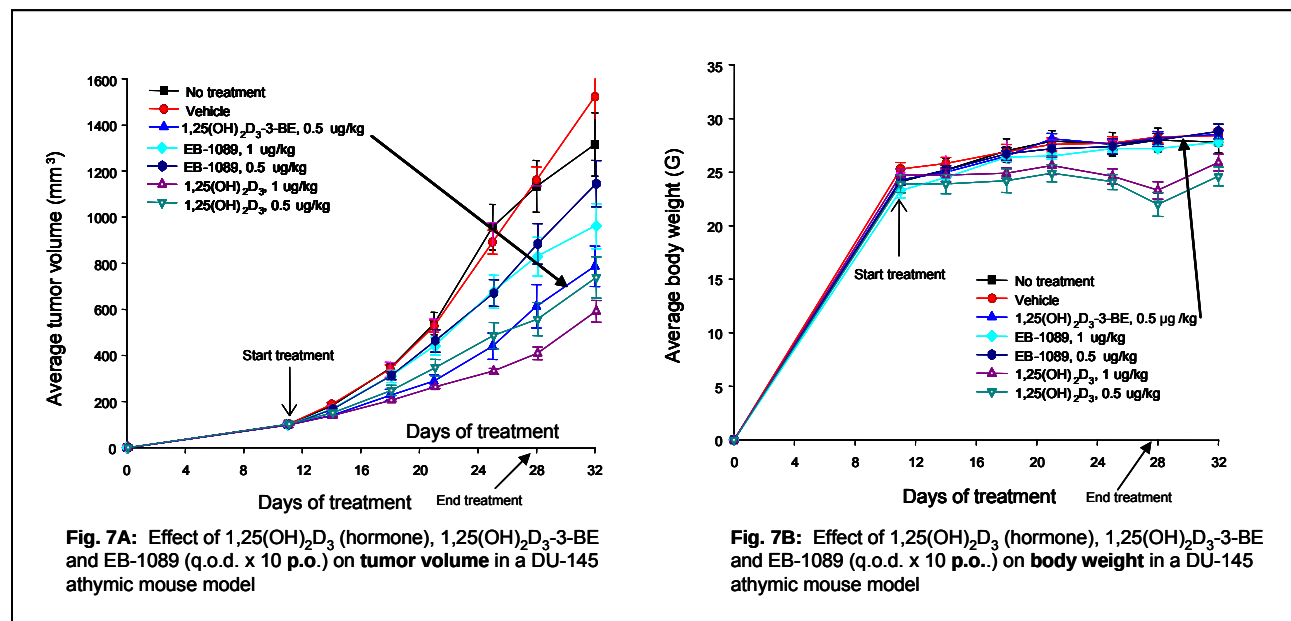
There are a few points to note in **Figures 6A & 6B**: (i) both 1,25(OH)₂D₃-3-BE, 1,25(OH)₂D₃ are more efficacious than EB-1089 (Seocalcitol), the most promising



1,25(OH)₂D₃-analog, (ii) 0.1 µg/kg of 1,25(OH)₂D₃-3-BE and 0.5 µg/kg of 1,25(OH)₂D₃ are approximately equivalent in efficacy. If we take into consideration the molecular weights of 1,25(OH)₂D₃ (416.65) and 1,25(OH)₂D₃-3-BE (537.8) 1,25(OH)₂D₃-3-BE is approximately 7-times more efficient in tumor-reduction than 1,25(OH)₂D₃ on a molar basis (as shown in **Figure 6C**). Moreover, 1,25(OH)₂D₃-3-BE was less toxic (as measured by reduction in body weight) than of 1,25(OH)₂D₃ as shown in **Figure 6B**.

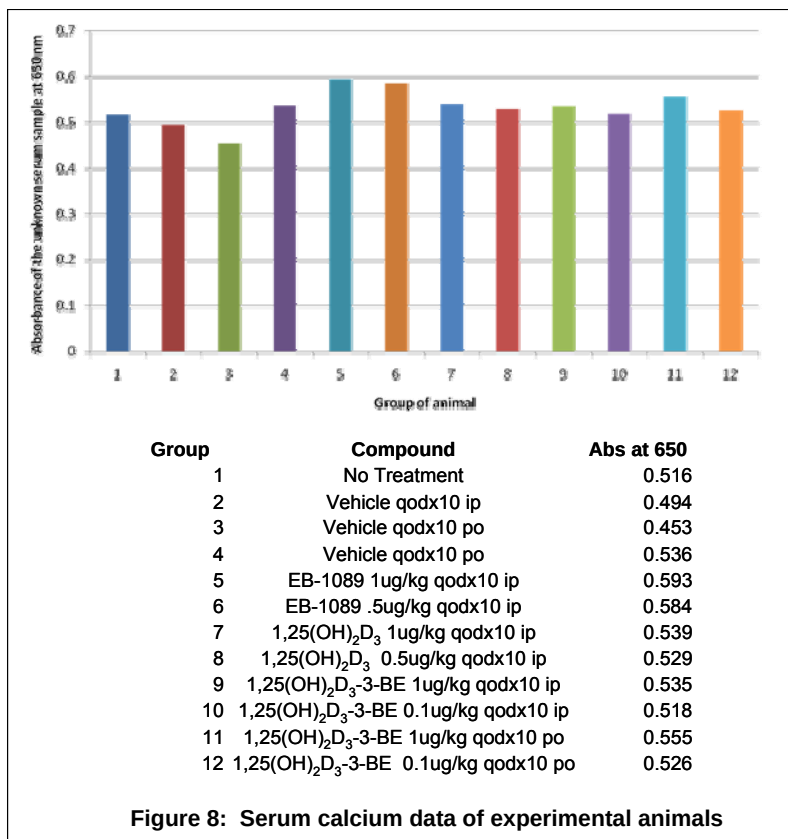
Results (*p.o.*-administration, Figure 7A & 7B):

As in *i.p.* administration mode, both 1,25(OH)₂D₃-3-BE, 1,25(OH)₂D₃ are more efficacious than EB-1089 (Seocalcitol). However, in contrast with the *i.p.* mode, 0.5 µg/kg 1,25(OH)₂D₃-3-BE and of 1,25(OH)₂D₃ caused approximately same extent of tumor reduction



(Figure 7A). However, 0.5 µg/kg of 1,25(OH)₂D₃-3-BE was not toxic at all, while 0.5 µg/kg 1,25(OH)₂D₃ caused significant toxicity as evidenced by reduction of body weight (Figure 7B).

Serum calcium measurement of blood samples:



Serum calcium is a measurement of calcemic toxicity. Therefore, we measured serum calcium levels of all the animals using a calcium measurement kit (Chemical Diagnostic Limited, Cat no. 140-20). As shown in Figure 8, serum calcium levels of all the treated groups were not significantly different from the untreated and vehicle-treated controls. These results indicate that vitamin D compounds have no residual toxicity after the drug-treatment was withdrawn (end treatment: 28 days, blood withdrawn: 32 days).

In summary, 1,25(OH)₂D₃-3-BE demonstrated strong tumor-reduction in both *i.p.* and *p.o.*

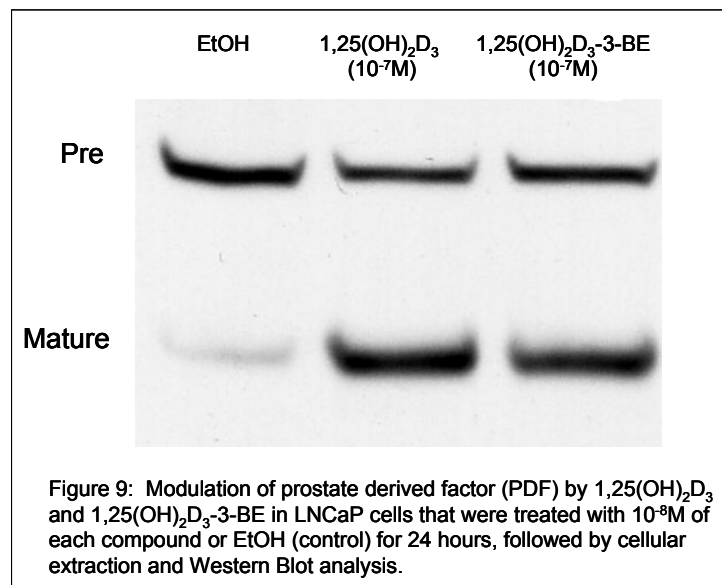
modes in a dose-dependent manner without significant toxicity in an androgen-insensitive prostate tumor athymic mouse model.

IV: Mechanistic studies of the 1,25(OH)₂D₃-3-BE in prostate cancer cells

Induction of Prostate Derived Factor (PDF) by 1,25(OH)₂D₃-3-BE in LNCaP cells

Prostate Derived Factor (PDF) is highly expressed in the prostate and prostatic expression of PDF is regulated by androgens. ***in situ* hybridization studies showed that PDF is expressed at high levels in normal prostate, down-regulated during progression of cancer at the primary site and re-expressed in osseous metastatic lesions but not lung, liver or lymph node lesions.** These data implicating PDF in the growth and differentiation state of prostate and other tissues, together with our preliminary data linking 1,25(OH)₂D₃ activity in

prostate cancer cells to PDF, suggest a role for PDF as a critical mediator of prostate homeostasis. Therefore, we investigated the role of 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE in inducing PDF in LNCaP prostate cancer cells.



Method: LNCaP cells (3.5x10⁵ cells/60 mm dish) were seeded for 16 hr before treatment with the indicated compounds for 24 hr, followed by re-suspending the cell pellets in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) containing protease inhibitors. After 10 min on ice, the extracts were centrifuged for

10 min, and protein concentration of each extract (supernatants) was determined (Bradford assay). 20 ug of each extract was separated on a 4-12% MES NuPAGE gel (Invitrogen) and transferred to PVDF membrane. PDF was detected with rabbit anti-PDF IgG. Western blots were developed using ECL. The control band represents a non-specific cross-reacting protein and serves as a loading control.

Results: PDF is synthesized as a pre-protein, cleaved and secreted as a 14 KD mature form, hence the upper and lower bands. Both 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE strongly induced PDF at 10⁻⁸M dose level (**Figure 9**). These results strongly suggest that 1,25(OH)₂D₃-3-BE is similar to 1,25(OH)₂D₃ in inducing PDF in LNCaP cells.

KEY RESEARCH ACCOMPLISHMENTS

- Conducted mouse xenograft studies for androgen-insensitive prostate tumor, demonstrating the efficacy of 1,25(OH)₂D₃-3-BE in reducing tumor-size in both i.p. and p.o. modes of administration.
- Demonstrated antiproliferative activity of 1,25(OH)₂D₃-3-BE in androgen-sensitive and androgen-insensitive prostate cancer cells in a dose-dependent manner.

- Demonstrated superiority of 1,25(OH)₂D₃-3-BE over 1,25(OH)₂D₃ and EB-1089, most promising non-calcemic vitamin D analog in inhibiting the proliferation of DU-145 androgen-insensitive prostate cancer cells.
- Carried out further studies to evaluate mechanistic aspects of the antiproliferative property of 1,25(OH)₂D₃-3-BE in prostate cancer cells.

REPORTABLE OUTCOME

During the past one year our efforts with 1,25(OH)₂D₃-3-BE and a related studies 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 cancer.

APPENDIX

Meeting Abstracts:

Evans Department of Medicine Research Days, Boston University School of Medicine, October 30-31, 2008

Abstract 1. 1,25-Dihydroxyvitamin D₃-3-bromoacetate, a novel vitamin D analog in pancreatic cancer. Susan Chadid, Vikram Jonathan Eddy, Kelly Persons, Asish Saha and Rahul Ray. Endocrinology, Diabetes and Nutrition Unit. Pancreatic cancer is largely resistant to standard chemo and radiation therapies. Moreover, development of drugs/combination of drugs for this disease is urgently needed. The hormonally active form of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) has shown strong promise as an antiproliferative agent in several malignancies. We have developed a novel derivative of 1,25(OH)₂D₃ (1 α ,25-dihydroxyvitamin D₃-3-bromoacetate, 1,25(OH)₂D₃-3-BE) that covalently attaches 1,25(OH)₂D₃ inside the ligand-binding pocket of vitamin D receptor that regulates the biological activities of 1,25(OH)₂D₃. We have employed thymidine incorporation and cell-count assays to determine the effects of 1,25(OH)₂D₃-3-BE in several pancreatic cancer cell lines. In addition, a combination of 1,25(OH)₂D₃-3-BE and 5-Aminoimidazole-4-carboxamide-1- β -4-ribofuranoside (AICAR), a widely used AMP-kinase (AMPK) activator, results in strong growth-inhibition of pancreatic cancer cells in a synergistic manner. In addition, we have carried out several pathway marker assays to evaluate the molecular mechanism of 1,25(OH)₂D₃-3-BE, either alone or with AICAR. Results of these studies will be discussed. Therefore, we conclude that 1,25(OH)₂D₃-3-BE either alone or in combination with AICAR has strong therapeutic potential in pancreatic cancer.

Abstract 2. Role of dietary vitamin D and calcium in an athymic mouse model of androgen-insensitive prostate cancer. Vikram J. Eddy, Hilal Abuzahra, Demetrios Vorgis, Kelly Persons, Michael F. Holick, Rahul Ray. Endocrinology, Diabetes & Nutrition Unit. Vitamin D deficiency has been associated with increased risk of prostate cancer. There has also been an association made between high calcium and increased prostate cancer risk. In this study we evaluated the effect of dietary vitamin D and calcium in a mouse model of androgen-insensitive prostate cancer. In the first arm (chemopreventive study) athymic mice were put on custom diets with varying levels of vitamin D and calcium for a month followed by tumor induction and monitoring of the tumors for an additional month. In the second arm (chemotherapeutic study), mice were fed normal chow and tumor was established to a certain size, then normal chow was switched to custom diets. Tumor-size was monitored for a month. In the chemopreventive arm, normal calcium/no vitamin D mice showed the highest rate of tumor growth. We are currently analyzing the data for the therapeutic study. Results of both the studies will be discussed in this poster. In conclusion, results of these studies will be extremely important in evaluating the role of dietary vitamin D and calcium in the prevention of prostate cancer and probable therapeutic effect in an established tumor.

Invited Lectures:

Nuclear transcriptional factors as molecular targets for drug discovery and delivery.
Northeastern University, Boston, MA, September 15, 2008

Signal transduction via Nuclear Receptors: Biochemistry, Structural Biology, and Therapeutic Targets.

Department of Molecular Medicine, Bose Institute, Kolkata, India, April 5, 2008.

Signal transduction via Nuclear Receptors: Biochemistry, Structural Biology, and Therapeutic Targets

Department of Biotechnology, University of Calcutta, Kolkata, India, April 24, 2008.

Cross-talk among structural domains of human DBP upon binding 25-hydroxyvitamin D

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Abstract

Serum vitamin D-binding protein (DBP) is structurally very similar to serum albumin (ALB); both have three distinct structural domains and high cysteine-content. Yet, functionally they are very different. DBP possesses high affinity for vitamin D metabolites and G-actin, but ALB does not. It has been suggested that there may be cross-talk among the domains so that binding of one ligand may influence the binding of others. In this study we have employed 2-*p*-toluidinyl-6-sulfonate (TNS), a reporter molecule that fluoresces upon binding to hydrophobic pockets of DBP. We observed that recombinant domain III possesses strong binding for TNS, which is not influenced by 25-hydroxyvitamin D₃ (25-OH-D₃), yet TNS fluorescence of the whole protein is quenched by 25-OH-D₃. These results provide a direct evidence of cross-talk among the structural domains of DBP.

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Keywords: Structural domains of vitamin D-binding protein (DBP); 25-Hydroxyvitamin D₃ (25-OH-D₃); 2-*p*-Toluidinyl-6-sulfonate (TNS); Reporter molecule; Conformational change; Cross-talk among domains

Vitamin D-binding protein (DBP) or group specific component (Gc) is a relatively abundant, polymorphic, and sparsely glycosylated serum protein with multiple functions. DBP binds vitamin D and its metabolites with high affinity ($K_d = 10^{8-11} \text{ M}^{-1}$); and this property (of DBP) is manifested in the organ-specific transportation of vitamin D and its metabolites to target tissues and stepwise oxidation of vitamin D₃ into its physiologically active metabolite, 1 α ,25-dihydroxyvitamin D₃ [1–3]. DBP also binds serum G-actin with high affinity ($K_d \approx 10^8 \text{ M}^{-1}$). Such an interaction is aided by plasma gelsolin and prevents G-actin from polymerizing into F-actin and blocking arteries under conditions of cellular injury or death. This property has serious implications in thrombosis and heart attack [4–8]. DBP also binds chemotactic agents such as C5a and C5a des Arg, thus enhancing complement activation on neutrophil chemotaxis [9,10]. In addition, DBP binds saturated and poly-unsaturated fatty

acids with high affinities [11,12]. Moreover, a post-translationally modified version of DBP (DBP-macrophage activating factor) has been shown to have strong macrophage- and osteoclast-activating [13–17], as well as antiangiogenic and anti-tumor properties [18,19].

DBP belongs to the albumin gene family; and it is structurally highly homologous with albumin (ALB), alpha-feto protein, and afamin [20–22]. All these proteins are characterized by modular structures with three structural domains (domains I–III) and high cysteine (Cys)-content. In the case of DBP domain III is considerably truncated compared with other members of this gene family. In addition, all the Cys residues in DBP, in contrast with ALB, are oxidized to disulfide linkages.

During the past decade several structure–function studies were carried out to strongly suggest that different domains of DBP are responsible for its various ligand-binding activities. For example, domain I was shown to be exclusively reserved for vitamin D sterol-binding [23–27], while G-actin-binding takes place in domain III [23,26,27]. DBP-*maf* activities, which are manifested by

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the partial deglycosylation of carbohydrate-containing DBP, are also restricted to domain III of the protein [28]. In the light of these observations it has been suggested that there may be a cross-talk among the structural domains of DBP so that binding of one ligand may influence the binding of another.

In this investigation we probed 25-hydroxyvitamin D₃ (25-OH-D₃)-binding (the strongest binder among all naturally occurring vitamin D metabolites) by human DBP using 2-*p*-toluidinyl-6-sulfonate (TNS) as a fluorescent reporter molecule. Results of the above studies are discussed in this communication in the light of multiple ligand-binding by DBP and its probable physiological implications.

Materials and methods

Purified human DBP was obtained from commercially available pooled human serum (American Red Cross, Dedham, MA) by a ligand affinity chromatographic method developed in our laboratory [29]. The recombinant C-terminal domain of hDBP (domain III, hDBP 277–458) was expressed in bacteria by our published procedure [25,30]. All other chemicals and biochemicals were obtained from Sigma–Aldrich Chemical Co., Milwaukee, WI, except [26(27)-³H]25-hydroxyvitamin D₃ (³H-25-OH-D₃, specific activity 20 Ci/mmol), that was purchased from DuPont–NEN, Boston, MA.

hDBP + TNS + 25-OH-D₃ (various amounts). Samples (20 µg) of hDBP in Tris buffer, pH 8.4, were incubated with TNS (2 µg) at 25 °C for 20 min. After this period, different amounts of 25-OH-D₃ (0.001, 0.01, 0.1, and 1 µg) were added to the DBP–TNS solutions, and fluorescence intensities were recorded in a Hitachi F 2000 Fluorescence Spectrophotometer. In a separate experiment, fluorescence spectra of TNS alone (in Tris buffer), TNS + 25-OH-D₃, hDBP + TNS, and hDBP + TNS + 25-OH-D₃ (1 µg) were recorded.

hDBP + ³H-25-OH-D₃ + TNS (various amounts). Samples of hDBP (20 µg each) in Tris buffer, pH 8.4, were incubated for 20 h at 4 °C with ³H-25-OH-D₃ (4000 cpm) either without or with various amounts of TNS (0.25–8.5 µg, as shown in Fig. 2). After the incubation, the samples were incubated on ice with Dextran-coated charcoal for 15 min. The samples were centrifuged (5000 rpm, 4 °C). Supernatant from each sample was mixed with scintillation cocktail and counted for radioactivity.

Competitive binding assay of hDBP with ³H-25-OH-D₃ and a fixed amount of TNS. Samples (20 µg) of hDBP were incubated at 4 °C for 20 h with ³H-25-OH-D₃ (4000 cpm) either with or without TNS (1.5 µg) and an increasing concentration of 25-OH-D₃ (0.05–51.2 ng, as shown in Fig. 3). Another set of samples without any TNS was treated the same way (control). The rest of the procedure is same as described earlier.

hDBP 277–458 + TNS + 25-OH-D₃. Samples of hDBP 277–458 (20 µg each) in Tris buffer, pH 8.4, were incubated with TNS (2 µg) at 25 °C for 20 min. After this period, 25-OH-D₃ (1 µg) was added to the solutions, and fluorescence spectra were recorded.

Results and discussion

DBP, similar to other members of albumin (ALB) gene family, has a triple-domain modular structure and a large number of cysteine (Cys) residues. All twenty-eight (28) Cys residues in DBP are engaged in forming fourteen (14) disulfide bonds leading to the formation of these domains. Domain I spans about 200 amino acids and stabilized by five disulfide bonds, and contains the only Trp (145) residue which is involved in vitamin D sterol-binding

[31]. Domain II is about one hundred and seventy-five (175) amino acids long and contains six (6) disulfide bonds. Domain III spans about eighty-five (85) amino acid residues (starting from amino acid residue 375) to the carboxy terminus and is stabilized by two (2) disulfide bonds.

ALB also has a triple-domain structure like DBP, but, in spite of high sequence and structural homology, DBP and ALB are functionally quite different. For example, DBP is a highly specific binder of vitamin D sterols and G-actin, while ALB is not. Moreover, DBP-*maf*-like activities of ALB are unknown to date. Accommodation of multiple high-specificity binders and multifunctional nature of DBP raises the possibility that binding of one ligand might influence the binding of other(s) via ‘cross-talk’ among interacting domains, and such a process might ultimately influence its functions. However, to date there has not been any direct evidence of such cross-talk among domains of DBP.

Changes in the intrinsic fluorescence of proteins (of aromatic amino acid residues) upon ligand/substrate-binding have been used quite extensively to study the micro-environment around these amino acids [32]. In addition, certain fluorescent hydrophobic molecules have been used as reporter molecules to probe micro-environment in proteins. 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) is such a molecule. TNS does not fluoresce in an aqueous solvent, but fluoresces strongly in non-polar organic solvents and when bound to hydrophobic regions of a protein. In some cases this binding is strongly influenced by the binding of the natural ligand/ligands. For example, TNS produces high quantum-yield fluorescence with serum ALB, beta-lactalbumin, and chymotrypsin, while with other proteins, like lysozyme, IgG, and ovalbumin, quantum-yields are considerably lower [32].

Goldschmidt-Clermont et al. showed that DBP displays strong fluorescence upon binding TNS, and this fluorescence is reduced in a dose-dependent manner by G-actin, and fluorescence is completely quenched at a concentration ratio of 1:1 [33]. Dose-dependent decrease in TNS fluorescence was explained as a representation of a change in conformation of DBP upon binding G-actin, instead of a simple displacement of TNS by G-actin. Such alteration in physicochemical properties has been reported in the literature. For example, binding between hemoglobin and haptoglobin has been shown to be accompanied by altered hydrophobicity and anodal shift in isoelectric focusing [34]. We carried out the present study to investigate the effect of 25-OH-D₃ on TNS-binding by hDBP.

Effect of 25-OH-D₃ on DBP–TNS fluorescence

We observed that TNS fluorescence decreased steadily with increasing concentration of 25-OH-D₃, and fluorescence intensity was almost completely obliterated by 1 µg of 25-OH-D₃ (Fig. 1). In support of this observation the strong hDBP–TNS-fluorescence peak at 435 nm (Fig. 1, inset, curve A) was almost completely obliterated by 25-

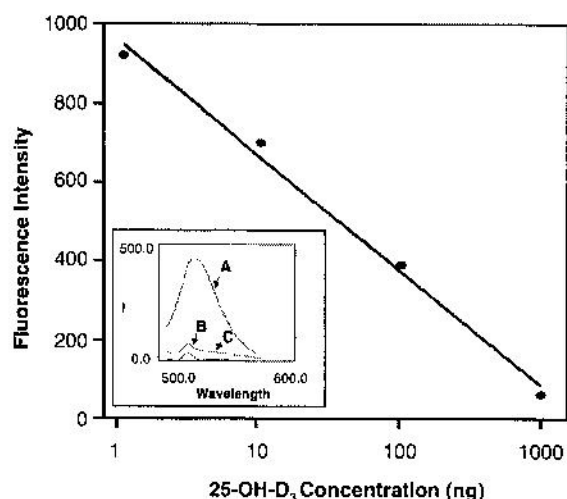


Fig. 1. TNS-fluorescence assays of human serum DBP (hDBP) in the presence of various amounts of 25-OH-D₃. (Inset) TNS-fluorescence spectra of hDBP + TNS (curve A), hDBP + TNS + 25-OH-D₃ (1 µg) (curve B), and TNS + 25-OH-D₃ (curve C).

OH-D₃ (1 µg) (Fig. 1, inset, curve B), while a combination of TNS and 25-OH-D₃ (1 µg) had very little fluorescence (Fig. 1, inset, curve C).

This dose-dependent decrease in TNS fluorescence by 25-OH-D₃ can be explained by either a direct competition between TNS and 25-OH-D₃ for binding site on DBP, or a change in physicochemical property (hydrophobicity, conformation) of DBP upon 25-OH-D₃-binding so that hydrophobic TNS-binding site/sites become progressively less available upon addition of 25-OH-D₃. To determine the mechanism of the above observation we carried out the following experiment.

Binding assay of hDBP with ³H-25-OH-D₃ in the presence of various amounts of TNS

Results of this assay show that ³H-25-OH-D₃-binding by DBP is not influenced at all by TNS (Fig. 2), strongly

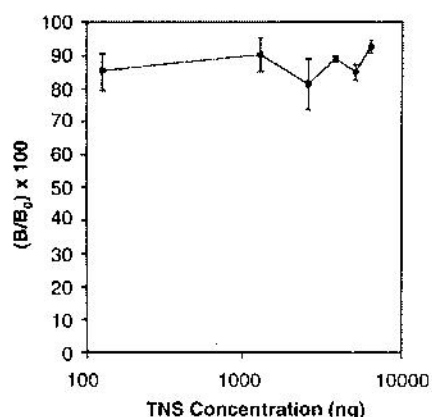


Fig. 2. ³H-25-OH-D₃ binding assay of hDBP in presence of various amounts of TNS.

suggesting that there is no direct competition between 25-OH-D₃ and TNS for binding site/sites in hDBP. However, it could not be ascertained (from these results) whether nature of 25-OH-D₃-binding to DBP (binding affinity) is altered by the binding of TNS. This was determined by the following experiment.

Assay to determine whether binding affinity of hDBP for 25-OH-D₃ is altered by TNS-binding

The competitive binding assay curves of DBP and ³H-25-OH-D₃ in the presence of 15 µg of TNS or in its absence are almost overlapping, indicating that TNS does not significantly alter interaction between 25-OH-D₃ and DBP qualitatively and quantitatively (Fig. 3).

Collectively the above results re-emphasize that there is no direct competition between 25-OH-D₃ and TNS for DBP-binding. In addition these studies indicate that TNS-binding does not alter the nature of binding between 25-OH-D₃ and DBP.

In the past our laboratory and others have shown that vitamin D sterol binding by DBP is largely restricted to domain I of the protein [23–25], while G-actin-binding takes place largely via domain III of DBP [23]. Furthermore, DBP–TNS fluorescence is reduced in a dose-dependent manner by G-actin, and is completely quenched at a concentration ratio of 1:1 [33], suggesting that TNS-binding might take place largely in domain III of DBP. In order to investigate that possibility we carried out TNS-binding by a recombinant domain III (mostly domain III and a small segment of domain II) of hDBP in the presence and in the absence of 25-OH-D₃.

Fluorescence spectra of recombinant hDBP 277–458 with TNS and 25-OH-D₃

We observed that hDBP 277–458 alone does not have any significant fluorescence activity (Fig. 4, curve B), but it displays strong fluorescence with a maximum at

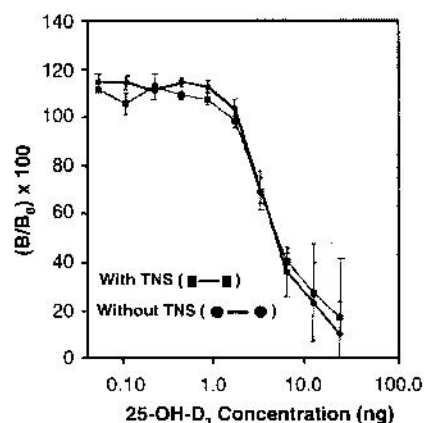


Fig. 3. Competitive binding assay of hDBP and ³H-25-OH-D₃ in the presence or in the absence of 15 µg of TNS.

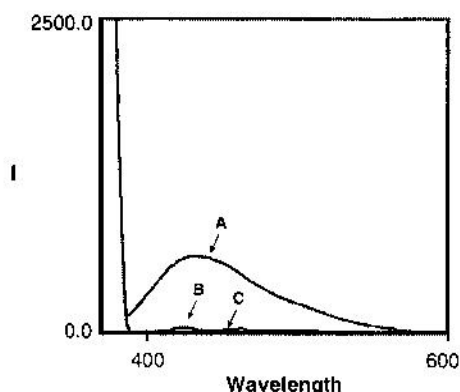


Fig. 4. TNS-fluorescence spectra of hDBP 277–458 peptide + TNS (curve A), hDBP 277–458 peptide alone (curve B), and hDBP 277–458 peptide + TNS + 25-OH-D₃ (curve C).

435 nm in the presence of TNS (Fig. 4, curve A, please note the change in the scale of the Y-axis from Fig. 1, inset). This peak was not at all influenced by the addition of an excess of 25-OH-D₃ (Fig. 4, curve C).

Collectively the above results suggest that major TNS-binding pocket in DBP may lie in domain III (C-terminal) of the protein. It could be argued that since domain III is not involved in 25-OH-D₃-binding [23–26], TNS fluorescence by this recombinant domain III is not influenced by 25-OH-D₃-treatment. However, this is in contrast with results displayed in Fig. 1 where we demonstrated that TNS fluorescence by full-length DBP is almost completely quenched by 25-OH-D₃. This apparent anomaly can be explained by significant conformational change in the whole protein upon 25-OH-D₃-binding (in domain I) to influence TNS-binding in domain III. As a result TNS fluorescence of full-length hDBP is quenched by 25-OH-D₃ in a dose-dependent manner (Fig. 1).

In summary, results of this study strongly imply that a considerable conformational change takes place in hDBP molecule upon binding 25-OH-D₃ in domain I of the protein; and this change is propagated into domain III to strongly influence TNS-binding in domain III of the protein, suggesting a cross-talk among the domains. DBP is a multi-functional protein. Therefore, this direct evidence of cross-talk has strong implications in the structure-functional aspects of this serum protein.

Acknowledgment

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Preliminary Communication

Fatty acid-binding site environments of serum vitamin D-binding protein and albumin are different

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Abstract

Vitamin D-binding protein (DBP) and albumin (ALB) are abundant serum proteins and both possess high-affinity binding for saturated and unsaturated fatty acids. However, certain differences exist. We surmised that in cases where serum albumin level is low, DBP presumably can act as a transporter of fatty acids. To explore this possibility we synthesized several alkylating derivatives of ¹⁴C-palmitic acid to probe the fatty acid-binding pockets of DBP and ALB. We observed that *N*-ethyl-5-phenylisooxazolium-3'-sulfonate-ester (WRK-ester) of ¹⁴C-palmitic acid specifically labeled DBP; but *p*-nitrophenyl- and *N*-hydroxysuccinimidyl-esters failed to do so. However, *p*-nitrophenyl ester of ¹⁴C-palmitic acid specifically labeled bovine ALB, indicating that the micro-environment of the fatty acid-binding domains of DBP and ALB may be different; and DBP may not replace ALB as a transporter of fatty acids.

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Keywords: Fatty acid-binding by vitamin D-binding protein (DBP) and albumin (ALB); Serum transport of fatty acids; Affinity labeling analogs of palmitic acid; Affinity labeling of fatty acid-binding sites of DBP and ALB

1. Introduction

Group specific component (Gc) or vitamin D-binding protein (DBP) is a sparsely glycosylated and polymorphic serum protein. The two major phenotypes are Gc1 and Gc2, differing from each other by four (4) amino acids in the primary structure as well as structure of attached polysaccharide. Gc1 is further divided into two subtypes differing in primary structure as well as structure of the attached carbohydrates [1–3].

DBP is a multi-functional protein [4]. Its binding of vitamin D and its metabolites has been studied extensively leading to the understanding that DBP is responsible for the stepwise activation of vitamin D₃ to 25-hydroxyvitamin D₃ (25-OH-D₃) and finally to its physiologically most active metabolite, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). It is also involved in the transportation of

these small molecules to organs and cells wherever they are required. In addition DBP plays an integral role in the circulating actin-scavenging system in plasma. Plasma gelsolin severes filaments of F-actin, and DBP binds to actin monomer (G-actin) with high affinity, thus preventing G-actin to polymerize and clog arteries during cell-injury and lysis [5,6]. Presence of actin–DBP complex in the sera of human and animals sustaining injuries/inflammation, e.g. trophoblastic emboli, severe hepatitis, acute lung injury, etc. positively implicates DBP in thrombosis and heart attack [7]. DBP also binds chemotactic agents such as C5a and C5a des Arg, thus enhancing complement activation on neutrophil chemotaxis [8,9]. Furthermore, a post-translationally modified form of DBP (DBP-macrophage activating factor, DBP-*maf*) has been shown to have strong macrophage- and osteoclast-activating [10–14] and anti-angiogenic and anti-tumor properties [15,16].

In addition to above properties of DBP and its derivative (DBP-*maf*), DBP binds saturated and unsaturated fatty acids with high affinity ($K_d = 10^5$ – 10^6 M^{−1}), similar to plasma ALB [17,18]. However, certain differences do

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exist. For example, Ena et al. demonstrated that molar ratio of fatty acids, bound to human DBP to DBP is 0.4 compared with 1.8 for human ALB [19]. Furthermore, majority of DBP-bound fatty acids are mono-unsaturated or saturated, and abundance of poly-unsaturated fatty acids is less than 5% (of the total bound fatty acids) [19]. Another interesting observation includes competition between vitamin D sterols and fatty acids in terms of binding to DBP. For example, it was reported that poly-unsaturated fatty acids, such as arachidonic or linoleic acid, strongly compete with 25-OH-D₃ and 1,25(OH)₂D₃ for binding to DBP, in sharp contrast with saturated fatty acids e.g. palmitic acid, which offer no significant competition [19,20]. Furthermore, Bouillon et al. observed that addition of human ALB in a physiological ALB:DBP ratio did not impair the inhibitory effect of linoleic acid towards DBP-25-OH-D₃-binding [20].

We hypothesized that this apparent anomaly between DBP and ALB in terms of fatty acid-binding might be related to the actual binding process between these proteins and fatty acids, which, in turn, might be related to the micro-environment of the fatty acid-binding pockets of these proteins. In order to evaluate this possibility we synthesized several reactive esters of ¹⁴C-palmitic acid as potential affinity labeling reagents for DBP and ALB. Results of these studies and their probable physiological implications are discussed in this report.

2. Materials and methods

Purified human DBP was obtained from commercially available pooled human serum (American Red Cross, Dedham, MA) by a ligand affinity chromatographic method developed in our laboratory [21]. Defatted bovine serum ALB (BSA) and all chemicals were purchased from Sigma–Aldrich, Milwaukee, WI, except 1-¹⁴C-palmitic acid (specific activity 56 mCi/mmol) which was a product of NEN-DuPont, Boston, MA.

2.1. Synthesis (Fig. 1)

The *N*-hydroxysuccinimido- and *p*-nitrophenyl-esters of palmitic acid were synthesized by dicyclohexylcarbodiimide (DCC)-coupling of palmitic acid with *N*-hydroxysuccinimide, or *p*-nitrophenol in the presence of a catalytic amount of *N,N'*-dimethylaminopyridine (DMAP) in anhydrous dichloromethane. Synthesis of WRK-palmitate was carried out by treating palmitic acid with *N*-ethyl-5-phenyl-isooxazolium-3'-sulfonate (Woodward's reagent K) and triethylamine in acetonitrile. Product from each reaction was purified by preparative chromatography on silica plates (Analtech, Vineland, NJ), and each product was characterized by NMR. Radioactive synthesis was carried out exactly the same way except palmitic acid was replaced with ¹⁴C-palmitic acid. Products from the radioactive reaction were isolated by TLC matching with corresponding unlabeled compounds.

2.2. Affinity labeling studies of bovine serum ALB and DBP with *N*-hydroxy-succinimido-¹⁴C-palmitate (A), *p*-nitrophenyl-¹⁴C-palmitate (B), and WRK-¹⁴C-palmitate (C)

Twenty-microgram samples each of BSA and DBP in 20 µl of TEST buffer (50 mM Tris–HCL, 150 mM NaCl, 1.5 mM EDTA, 0.1% Triton X-100, pH 8.8) were treated with *N*-hydroxysuccinimido-¹⁴C-palmitate (A), *p*-nitrophenyl-¹⁴C-palmitate (B), or WRK-¹⁴C-palmitate (C) (each 20,000 cpm) at 25 °C for 20 h. Parallel samples of BSA and DBP containing additional sodium palmitate (1 µg in 10 µl of buffer) were also treated the same way. At the end of the experiment all the samples were analyzed on a 7.5% SDS–polycarylamide gel, followed by drying the gel and scanning of radioactivity in a Biosan phosphorimager.

3. Results and discussion

There is a remarkable structural homology among ALB, DBP, α-feto protein (AFP) and afamin, members of the albumin gene family. All these proteins have modular structures with three domains (domains I–III) and high cysteine-content [22]. In the case of DBP all the Cys residues (total 28) are oxidized to form 14 disulfide bonds. In contrast, ALB contains several free sulphhydryl groups in its primary structure. Furthermore, DBP has a shorter domain III than ALB. These structural differences may explain gross functional differences between DBP and ALB. For instance, vitamin D sterols- and G-actin binding and related functions are unique to DBP. On the other hand, DBP possesses relatively weaker binding for fatty acids compared with ALB. Furthermore, DBP contains a single high-affinity fatty acid-binding site compared to ALB which contains several low- and high-affinity binding sites [18]. In ALB these binding sites are distributed among various domains of the protein, although high-affinity-binding sites are located in domain III [23]. Moreover, as described earlier, DBP, in contrast with ALB, discriminates between saturated and unsaturated fatty acids in terms of binding.

All the above observations point to difference in the nature of binding between ALB and DBP and fatty acids, which in turn may be related to the fatty acid-binding pocket structure of these proteins. Affinity and photo-affinity labeling techniques have been used widely to probe binding pockets and catalytic active sites of receptors and enzymes, respectively [24]. Our laboratory has used these techniques, and others to probe the vitamin D and actin-binding domain structures of DBP, leading to crystal structure of the DBP–actin complex [25–35].

In the current study we synthesized radiolabeled versions of three reactive esters of palmitic acid to probe the fatty acid-binding pockets of DBP and ALB. We chose palmitic acid, a saturated fatty acid as model because DBP has a propensity to bind saturated and mono-unsaturated fatty acids stronger than poly-unsaturated fatty acids [19,20].

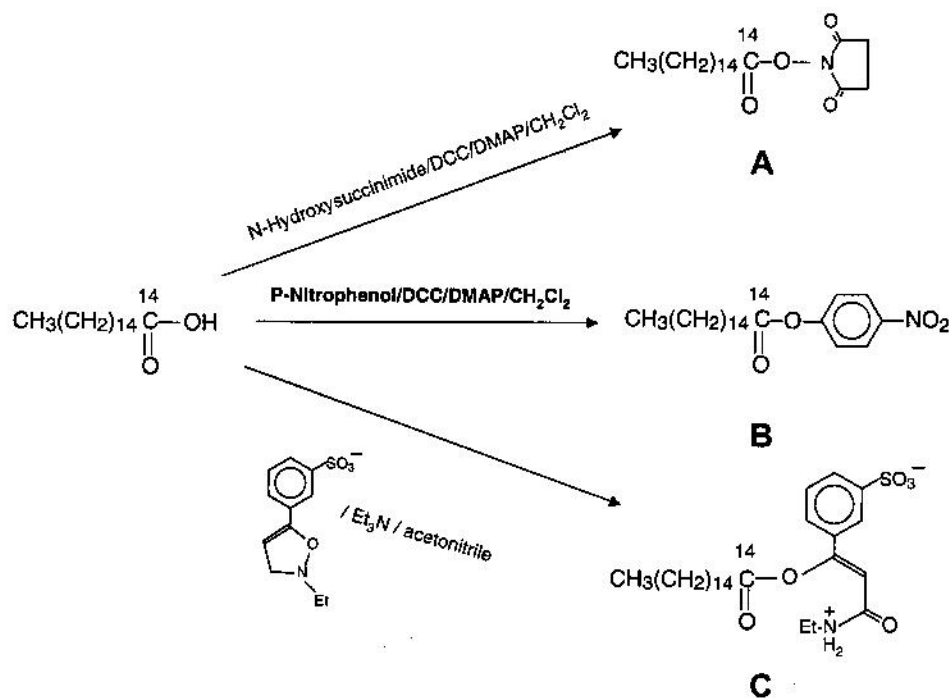


Fig. 1. Scheme for the synthesis of *N*-hydroxy-succinimido- ^{14}C -palmitate (A), *p*-nitrophenyl- ^{14}C -palmitate (B), and WRK- ^{14}C -palmitate (C).

Reed employed WRK- ^{14}C -palmitate (C) to affinity label the fatty acid-binding pocket/s of bovine serum ALB [36]. In our case, incubation of a sample of human serum DBP (hDBP) with WRK- ^{14}C -palmitate (C) covalently labeled the protein as determined by autoradiography (Fig. 2, lane 1). When the incubation was carried

out in the presence of an excess of sodium palmitate, labeling was completely obliterated (Fig. 2, lane 2). These results strongly indicated that WRK- ^{14}C -palmitate (C) specifically labeled the palmitic acid-binding pocket in hDBP. These results also suggested that structure and chemical environment of the fatty acid-binding pocket of DBP and ALB are similar.

Surprisingly other activated esters of palmitic acid i.e. *N*-hydroxysuccinimidyl- ^{14}C -palmitate (A) and *p*-nitrophenyl- ^{14}C -palmitate (B) failed to label DBP in the presence or in the absence of an excess of sodium palmitate. In the case of BSA, *N*-hydroxysuccinimidyl- ^{14}C -palmitate (A) failed to label this protein. But, *p*-nitrophenyl- ^{14}C -palmitate (B) labeled BSA, and labeling was significantly reduced in the presence of an excess of palmitic acid, denoting specific labeling of the fatty acid-binding pocket (results not shown).

Collectively the above results suggest that chemical/electronic environments of the fatty acid-binding pockets of DBP and ALB are different, so that ALB can tolerate a hydrophobic (*p*-nitrophenyl) as well as a hydrophilic (Woodward K reagent) head group at the carboxy terminus of palmitic acid. But, fatty acid-binding site of DBP can only accommodate a polar and Zwitterionic head group (Woodward K reagent).

Analbuminemia is a rare hereditary disease in which the afflicted individuals have very low or negligible amount of circulating serum ALB [37–39]. We surmised that since both ALB and DBP bind fatty acids with high affinity DBP may replace ALB in carrying fatty acids, particularly saturated and mono-unsaturated fatty acids in the cases of low or negligible amount of circulating ALB. However

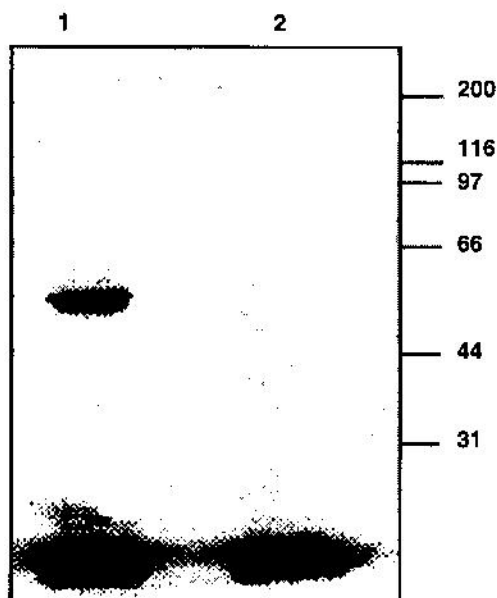


Fig. 2. Affinity labeling of hDBP with WRK- ^{14}C -palmitate (C): samples of hDBP were incubated at 25 °C with WRK- ^{14}C -palmitate (C) alone (lane 1), or in the presence of an excess of sodium palmitate (lane 2). The samples were electrophoresed on a SDS gel and exposed to a phosphor-imager. Positions of the standard molecular weight markers are denoted on the right.

results of the study delineated in this communication suggest that chemical and electronic environment of the fatty acid-binding pockets of DBP and ALB might be different. As a result binding and transportation of various fatty acids might be different. Thus, DBP may not replace ALB in terms of fatty acid scavenging and transportation.

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