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1α,25-Dihydroxyvitamin D_3-3β-bromoacetate, a potential cancer therapeutic agent: Synthesis and molecular mechanism of action

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Anti-cancer agent
Mechanism of action of $1,25(OH)_2D_3$-3-BE
1α,25-Dihydroxyvitamin D_3-24-hydroxylase (CYP24)

ABSTRACT

Synthesis of 1α,25-dihydroxyvitamin D_3-3β-bromoacetate ($1,25(OH)_2D_3$-3-BE), a potential anti-cancer agent is presented. We also report that mechanism of action of $1,25(OH)_2D_3$-3-BE may involve reduction of its catabolism, as evidenced by the reduced and delayed expression of 1α,25-dihydroxyvitamin D_3-24-hydroxylase (CYP24) gene in cellular assays.

The awareness of the potential beneficial effect of vitamin D in a number of diseases, and general good health has increasingly become a public health issue.[1] In addition, the therapeutic potential of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the dihydroxylated metabolite of vitamin D_3, on established tumors was realized when the potent antiproliferative property of $1,25(OH)_2D_3$ was discovered in the early 1980's in leukemic cells.[2] Since then the antiproliferative and pro-differentiative effects of $1,25(OH)_2D_3$ have been studied in various cancer cell lines, and as a result, $1,25(OH)_2D_3$ has been investigated as a potential anti-cancer agent.[3] However, clinical trials have demonstrated strong calcemic and calciuric effects at pharmacological doses (of $1,25(OH)_2D_3$).[4] This limitation provided the search for structural analogs of $1,25(OH)_2D_3$ with potent antiproliferative yet low calcemic properties.[4] This effort, based solely on a Specific Estrogen Receptor Modulator (SERM)-like concept adopted the idea that these analogs would change the conformation of nuclear vitamin D receptor (VDR), the chief modulator of the biological actions of $1,25(OH)_2D_3$ sufficiently from the parent hormone to separate cell-regulatory properties from calcemic property. Numerous such 'non-calcemic' analogs (of $1,25(OH)_2D_3$) have been synthesized based on this concept with the hope of achieving a favorable antiproliferative/toxicity index, yet clinical results are largely disappointing.[5,6]

On the other hand, recent clinical trials of $1,25(OH)_2D_3$ either alone or in combination with common cancer therapeutic drugs have produced strongly encouraging results, and brought back the realization that $1,25(OH)_2D_3$ is probably the most promising 'drug' if its therapeutic dose can be escalated without causing toxicity.[7–10] High doses of $1,25(OH)_2D_3$ are required to counter its non-tumor specific spread, and high catabolic rate. Realizing this unmet potential of $1,25(OH)_2D_3$-therapy, we adopted a hypothesis-based alternate approach to increase the half-life of $1,25(OH)_2D_3$, to thus decrease effective dose with reduced toxicity.

1α,25-Dihydroxyvitamin D_3-3β-bromoacetate ($1,25(OH)_2D_3$-3-BE) was initially developed in our laboratory as an affinity labeling reagent to map the ligand-binding domain (LBD) of VDR.[11] Subsequently, we realized that since $1,25(OH)_2D_3$-3-BE covalently attaches to VDR-LBD,[12–15] it might be physically protected from interacting with catabolic enzymes, thereby increasing its half-life. Furthermore, once attached to the VDR-LBD, $1,25(OH)_2D_3$-3-BE becomes a simple 3-acetate derivative of $1,25(OH)_2D_3$, activating gene transcription similar to $1,25(OH)_2D_3$. In other words, $1,25(OH)_2D_3$-3-BE becomes de facto $1,25(OH)_2D_3$ with an increased

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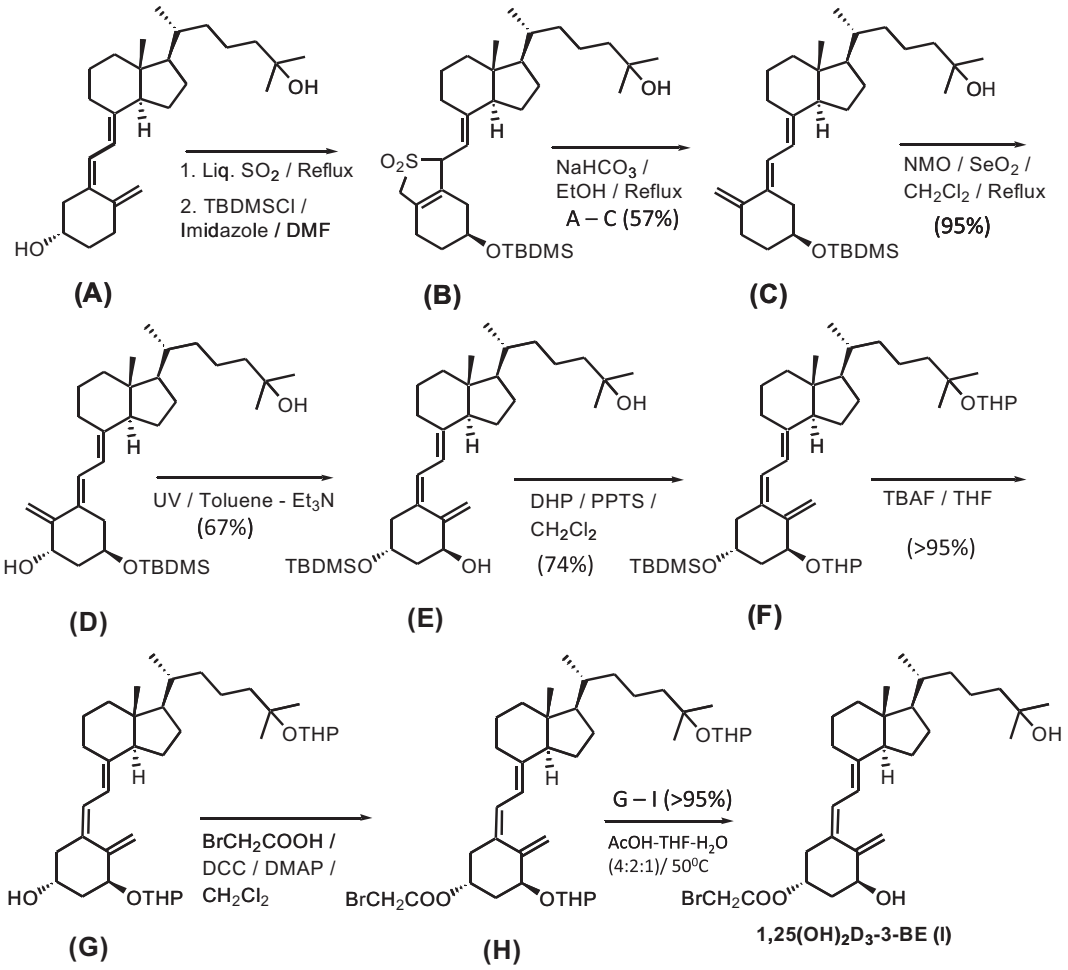
half-life by this process. We observed that $1,25(OH)_2D_3$-3-BE has a significantly greater antiproliferative effect than $1,25(OH)_2D_3$ in normal human keratinocytes, supporting our hypothesis.[16] Subsequently several publications from our group and others have demonstrated significantly stronger antiproliferative effect of $1,25(OH)_2D_3$-3-BE and its mono-hydroxylated analog, 25-hydroxyvitamin D_3-3β-bromoacetate than $1,25(OH)_2D_3$ in various cancer cells.[17–22] We also observed strong anti-tumor effect of $1,25(OH)_2D_3$-3-BE in mouse model of renal cancer,[22] and androgen-insensitive prostate cancer (manuscript in preparation).

The above mentioned results suggest strong therapeutic potential of $1,25(OH)_2D_3$-3-BE, and warrant extensive in vitro and in vivo studies, requiring substantial quantity of $1,25(OH)_2D_3$-3-BE. Previously, we reported synthesis of $1,25(OH)_2D_3$-3-BE with a starting material that is no longer available[23] mandating an alternative synthetic procedure for this compound, which is delineated in this Letter. Furthermore, our hypothesis about increased stability of $1,25(OH)_2D_3$-3-BE by making it less available to catabolic enzymes remains unproven to date. In this Letter, we report results of cellular studies to support this hypothesis on the catabolism of this analog.

Synthesis of $1,25(OH)_2D_3$-3-BE was initiated by converting 25-hydroxyvitamin D_3 (**A**, Duphar Chemicals, Netherlands) to its trans-variety (**B**) by the sulfur-dioxide method[24] and converting the crude into the *tert*-butyldimethylsilylether (TBDMS) derivative (**C**) (Scheme 1) in good yield.

Success of our synthetic scheme depended critically on the introduction of the 1-hydroxyl group into (**C**) by allylic oxidation in a stereospecific manner. In an earlier publication,[25] we reported that *N*-methylmorpholine-*N*-oxide/SeO_2allylic oxidation of 25-hydroxy-5*E*-[6,19,19′-H_3]vitamin D_3-3β-*tert*-butyldimethylsilyl ether to 1α,25-dihydroxy-5*E*-[6,19,19′-H_3]vitamin D_3-3β-*tert*-butyldimethylsilyl ether produced an approximately 6:1 ratio of 1α-OH:1β-OH stereoisomers. However, in the present case, HPLC analysis of the crude reaction mixture showed the product to be homogeneous, indicating the presence of a single stereoisomer (**D**) in the reaction mixture, which, after purification, was identified to have the 1α-OH (desired) stereochemistry by comparison with the NMR spectrum of a known sample.[25] We speculate that the lack of three deuterium atoms in the triene system adjoining the A-ring in **C** may have changed the conformation of the A-ring substantially to have the oncoming oxygen atom introduced exclusively from one side. We also found that stereochemistry at the 1-position depended on the structure of the 1-silyl ether. For example, when 1-OH was derivatized with a triethylsilyl group, the 1α-OH:1β-OH ratio was approximately 4:1 with an overall yield of 45%.

In the next step 1α,25-dihydroxy-5*E*-vitamin D_3-3β-*tert*-butyldimethylsilyl ether (**D**) was converted to its 5*Z* counterpart (**E**) by photolysis. Attempts to protect the 1-hydroxyl group in **E** as tetrahydropyranyl (THP) ether resulted in the derivatization of the 25-hydroxy group as well, and produced 1α,25-di-tetrahydropyranyloxy, 3β-*tert*-butyldimethysilyl-5*Z*-vitamin D_3 (**F**), which was de-silylated to produce 1α,25-di-tetrahydropyranyloxy-5*Z*-vitamin D_3 (**G**). Finally, **G** was DCC-coupled to bromoacetic acid, and the resulting product was de-protected to produce



Scheme 1. Scheme for the synthesis of $1,25(OH)_2D_3$-3-BE.

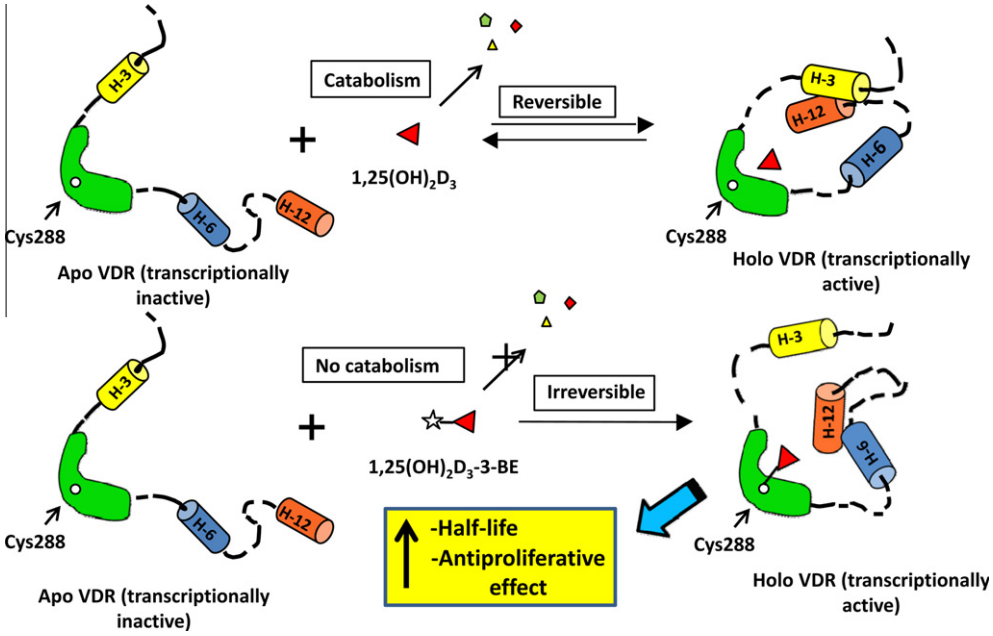


Figure 1. Cartoon depicting interaction between $1,25(OH)_2D_3$, $1,25(OH)_2D_3$-3-BE and VDR. Points to note: (i) reversible and irreversible nature of interaction of $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE, respectively, with VDR, leading to different catabolic outcomes, (ii) covalent attachment of $1,25(OH)_2D_3$-3-BE via Cys_{288} in VDR-LBD, (iii) differential conformational changes of VDR-LBD induced by $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE.

1α,25-dihydroxy-5Z-vitamin D_3-3β-bromoacetate (**I**, $1,25(OH)_2D_3$-3-BE) in nearly quantitative yield.

Signal transduction via $1,25(OH)_2D_3$ is a stepwise process that is initiated by the highly specific binding between $1,25(OH)_2D_3$ and VDR. Ligand binding allosterically promotes formation of a heteromeric complex with RXR, which allows the complex to bind to vitamin D response element(s) in chromatin and recruitment of co-activators, leading to transcription of vitamin D target genes. According to this dogma, VDR-binding by $1,25(OH)_2D_3$ as well as its analogs/derivatives such as $1,25(OH)_2D_3$-3-BE is crucial for all these processes.

$1,25(OH)_2D_3$-3-BE is a VDR affinity alkylating agent. In an earlier study, we described that $1,25(OH)_2D_3$-3-BE is capable of specifically labeling native VDR in ROS 17/2.8 bone cells and calf thymus nuclear extract,[12] and rapidly titrating recombinant VDR with 1:1 stoichiometry.[16] We also reported that $1,25(OH)_2D_3$-3-BE covalently labels a single cysteine residue (Cys_{288}) in the VDR-LBD (as shown in the cartoon in Fig. 1).[14] Furthermore, we demonstrated that VDR is essential for the growth inhibitory activity of 25-hydroxyvitamin D_3-3-BE, a prototype of $1,25(OH)_2D_3$-3-BE without the 1-hydroxyl group in ALVA-31 prostate cancer cells.[19]

The other mechanistic aspect of our hypothesis delineates that $1,25(OH)_2D_3$-3-BE, after interacting with VDR, is protected from catabolism, thus increasing its half-life, and enhancing its anti-growth property as evidenced in several studies.[17–22] 1α,25-Dihydroxyvitamin D_3-24-hydroxylase (CYP24) is a cytochrome P450 enzyme that introduces a hydroxyl group at the 24-position in the side chain of $1,25(OH)_2D_3$ that initiates the multi-step catabolic degradation.[26] Interaction between VDR and $1,25(OH)_2D_3$ is an equilibrium process so that the steady state would always contain 'free' $1,25(OH)_2D_3$ (not bound to VDR) which will be rapidly catabolized by CYP24 (Fig. 1).

In contrast, covalent attachment of $1,25(OH)_2D_3$-3-BE inside the ligand-binding pocket of VDR[14] would eliminate/reduce 'free' $1,25(OH)_2D_3$ in the steady state, thereby potentially requiring reduced and delayed expression of CYP24 (Fig. 1).

We chose A498 kidney cancer cells to test our hypothesis. In the dose–response experiment A498 cells were treated with 10^{-6} M of $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE or ethanol vehicle (control). As shown in Figure 2, treatment of A498 cells with 10^{-6} M of $1,25(OH)_2D_3$ strongly induced CYP24 mRNA expression. $1,25(OH)_2D_3$-3-BE treatment also led to induction of CYP24 mRNA, but at a significantly higher concentration (approximately 10-fold) to achieve induction comparable to $1,25(OH)_2D_3$, suggesting $1,25(OH)_2D_3$-3-BE is less efficacious in inducing CYP24 than $1,25(OH)_2D_3$, as predicted by our hypothesis.

In the kinetic experiment cells were treated with 10^{-8} M of either $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE and CYP24 message was detected at various time-points. As shown in Figure 3, induction of CYP24 mRNA was detectable by 4 h in $1,25(OH)_2D_3$-treated cells and continued to increase over 16 h. In contrast, $1,25(OH)_2D_3$-3-BE showed a much delayed kinetics of induction of CYP24 mRNA, with CYP24 mRNA first being detectable at 16 h.

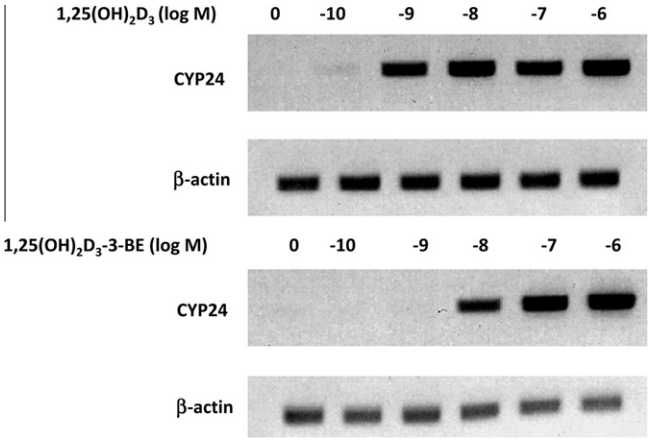


Figure 2. Comparison of the dose dependent increase of CYP24 mRNA by $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE. A498 cells were treated with ethanol control or 10^{-6} M of $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE for 24 h, total RNA was prepared and CYP24 mRNA levels assessed by RT-PCR. β-Actin mRNA levels were determined for each sample as control. The results are representative of two independent experiments.

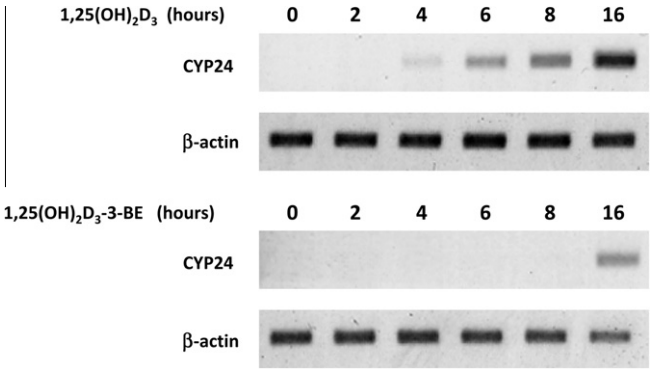


Figure 3. Kinetics of CYP24 message induction by $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE. A498 cells were treated with 10^{-8} M of $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE or ethanol control for 16 h, total RNA was prepared at specified times and CYP24 mRNA levels assessed by RT-PCR. β-Actin mRNA levels were determined for each sample as control. The results are representative of two independent experiments.

Taken together, results of Figures 2 and 3 suggest that $1,25(OH)_2D_3$-3-BE is less effective in inducing CYP24 gene expression than an equivalent amount of $1,25(OH)_2D_3$. CYP24 is a catabolic enzyme that initiates the degradation of $1,25(OH)_2D_3$. Therefore, reduced and delayed expression of CYP24 gene implies decreased catabolism of $1,25(OH)_2D_3$-3-BE, as predicted by our hypothesis.

We reported earlier that $1,25(OH)_2D_3$-3-BE changes the conformation of VDR differently from $1,25(OH)_2D_3$ as reflected in the enhanced stabilization of VDR-hOCVDRE (human osteocalcin vitamin D response element) complex in COS-1 cells and promotion of a longer stimulation of CYP24 mRNA expression in keratinocytes compared with $1,25(OH)_2D_3$.[16] Furthermore, recently we reported that compounds similar to $1,25(OH)_2D_3$-3-BE with alkylating bromoacetate group at 1- and 11-positions of $1,25(OH)_2D_3$ specifically labeled VDR-LBD, yet their antiproliferative activity in keratinocytes was similar to that of $1,25(OH)_2D_3$.[27] Therefore, considering all the information we ascribe enhanced antiproliferative activity of $1,25(OH)_2D_3$-3-BE to a combination of its increased half-life (by covalent labeling of VDR-LBD) and a unique change in VDR-conformation upon binding (Fig. 1).

Several non-genomic pathways for the action of $1,25(OH)_2D_3$ and its analogs have also been reported.[28] We have observed that PI3K/Akt non-genomic pathway is involved in the activity of $1,25(OH)_2D_3$-3-BE in kidney cancer cells.[22] Therefore, mechanism of action of $1,25(OH)_2D_3$-3-BE may involve a combination of genomic and non-genomic pathways.

In conclusion, we report an efficient synthesis of $1,25(OH)_2D_3$-3-BE, a potential therapeutic agent for cancer. In addition, we provide data from cellular studies to support our hypothesis about its intrinsic mechanism of action.

Acknowledgments

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Notes

Synthesis: (i) A mixture of (**C**) (760 mg) and SeO_2 (192 mg) in 15 ml of anhydrous CH_2Cl_2 was refluxed under argon for 30 min followed by cooling to room temperature and addition of a solution of *N*-methylmorpholine-*N*-oxide (850 mg) in 15 ml of anhydrous CH_2Cl_2. The mixture was refluxed for an additional 60 min. After usual workup and purification trans-1α,25-dihydroxyvitamin D_3-3β-TBDMS ether (**D**) was obtained in 95% yield.

(ii) A toluene (10 ml) solution of **D** (80 mg), anthracene (10 mg), Et_3N (40 μl) in a quartz test tube was irradiated from a Hanovia medium pressure mercury arc lamp for 75 min. After preparative TLC purification 1α,25-dihydroxyvitamin D_3-3-TBDMS ether (**E**) was obtained in 67% yield.

Cellular studies: (i) Induction of 1α,25-dihydroxyvitamin D_3-24hydroxylase (CYP24) gene expression by $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$-3-BE (dose–response): A498 kidney cancer cells (ATCC, Manasas, VA) were treated with 10^{-6} M of $1,25(OH)_2D_3$, $1,25(OH)_2D_3$-3-BE or ethanol (vehicle control) for 24 h. Total RNA was prepared and subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase and random hexamers under standard conditions. Following cDNA synthesis, PCR was performed using gene specific primers to the vitamin D target gene, CYP24 and β-actin (control). The products were analyzed on 1% agarose gels.

(ii) Induction of CYP24 gene expression by $1,25(OH)_2D_3$-3-BE (kinetics): In this experiment, A498 cells were treated with 10^{-8} M of $1,25(OH)_2D_3$-3-BE and $1,25(OH)_2D_3$ and RNA prepared over the course of 16 h for the analysis of CYP24 mRNA levels by RT-PCR.