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1α,25-Dihydroxyvitamin D_3-3β-(2)-Bromoacetate, an Affinity Labeling Derivative of 1α,25-Dihydroxyvitamin D_3 Displays Strong Antiproliferative and Cytotoxic Behavior in Prostate Cancer Cells

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Abstract In this report we describe that $1,25(OH)_2D_3$-3-BE, a VDR-affinity labeling analog of $1,25(OH)_2D_3$, showed strong and dose-dependent growth-inhibitory effect in several epithelial cells, i.e., keratinocytes (primary cells), MCF-7 breast cancer, PC-3, and LNCaP prostate cancer and PZ-HPV-7 immortalized normal prostate cell-lines. Furthermore, 10^{-6} M of $1,25(OH)_2D_3$-3-BE induced apoptosis specifically in LNCaP and PC-3 cells; and the effect was much less pronounced at lower doses. We also showed that the effect (of $1,25(OH)_2D_3$-3-BE) was not due to probable degradation (hydrolysis) of $1,25(OH)_2D_3$-3-BE or random interaction of this molecule with cellular proteins. Tissue- or cell-specific action of $1,25(OH)_2D_3$ and its mimics is not common due to the ubiquitous nature of VDR. Furthermore, variable effects of $1,25(OH)_2D_3$ and its analogs in various cell-lines potentially limits their application as anticancer agents. We showed that $1,25(OH)_2D_3$-3-BE displayed similar growth-inhibitory and cytotoxic activities towards androgen sensitive LNCaP and androgen-independent PC-3 cell-lines. Therefore, these results raise the possibility that $1,25(OH)_2D_3$-3-BE or similar VDR-cross linking analogs of $1,25(OH)_2D_3$ might be considered for further development as potential candidates for prostate cancer. J. Cell. Biochem. 89: 909–916, 2003.

Key words: 1,25-Dihydroxy vitamin D_3-Analog; vitamin D receptor; prostate cancer; affinity labeling; apoptosis; cell-specific effect

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, vitamin D hormone) and several of its synthetic analogs are known to modulate the growth of a number of cancer cells in vitro to prevent their proliferation and promote differentiation [Blutt and Weigel, 1999; Zhao and Feldman, 2001; Johnson et al., 2002; Van Den Bemd and Chang, 2002]. Such cell-regulatory properties of $1,25(OH)_2D_3$ and its agonists are associated with their specific binding to nuclear $1,25(OH)_2D_3$ receptor (nuclear vitamin D receptor, VDR) with subsequent activation of vitamin D-controlled genes in a multi-step process [Haussler et al., 1998; Jones et al., 1998]. Therefore, VDR is a rational molecular target for intervention by analogs of $1,25(OH)_2D_3$ in harnessing their potential anti-neoplastic properties.

During the past few years, we have developed a class of steroidal and non-steroidal analogs of $1,25(OH)_2D_3$ which covalently label the hormone-binding pocket of VDR [Ray et al., 1994; 1996; Swamy et al., 1997a; Chen et al., 1999; Swamy et al., 2000; Mohr et al., 2001; Fernandez-Gacio et al., 2003]. We demonstrated that 1,25-dihydroxyvitamin D_3-3β-(2)-bromoacetate ($1,25(OH)_2D_3$-3-BE), an affinity labeling analog of $1,25(OH)_2D_3$, cross-linked to the hormone-binding pocket of VDR; and such a process modulated the transcription of VDR-regulated genes. Level of transcription was higher with this analog than an equimolar

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amount of $1,25(OH)_2D_3$ for a longer duration [Chen et al., 1999]. Additionally, $1,25(OH)_2D_3$-3-BE displayed stronger antiproliferative activity in skin cells (keratinocytes) than $1,25(OH)_2D_3$ at various doses [Chen et al., 1999].

In the present study, we compared antiproliferative effects of $1,25(OH)_2D_3$-3-BE and $1,25(OH)_2D_3$ in a few normal and malignant epithelial cell lines, and observed that $1,25(OH)_2D_3$-3-BE displayed strongest growth-inhibitory effect in prostate cancer cells. We also made the serendipitous observation that this $1,25(OH)_2D_3$-mimic induced cytotoxicity specifically to prostate cancer cells. Results of these studies and their implications are discussed in this communication.

MATERIALS AND METHODS

$1,25(OH)_2D_3$-3-BE was synthesized according to a procedure developed in our laboratory [Ray et al., 1994]. Majority of the chemicals were purchased from Sigma-Aldrich, St. Louis, MO, unless mentioned otherwise. Cell-lines were obtained from ATCC (Rockville, MD).

Cell-Culture

All the cells were cultured by procedures recommended by the supplier, except in the case of keratinocytes and PZ-HPV-7 cells (see below). MCF-7, PC-3, and LNCaP cells were grown in DMEM media containing antibiotics and 5% fetal bovine serum (FBS) and antibiotics. PZ-HPV-7 cells were grown in MCDB media containing pituitary extract, EGF, and antibiotics. Keratinocytes were also grown in the same media (as PZ-HPV-7) with additional PG1 and insulin. In general, cells were grown in 35 mm dishes to 70–80% confluence, and then plated into 24-well plates in respective media. In general, steroid samples were dissolved in ethanol (EtOH), and dilution with the media was adjusted in such a way that concentration of EtOH was 0.1%. Growth-inhibition in these cells in response to $1,25(OH)_2D_3$-3-BE, $1,25(OH)_2D_3$, or EtOH control was determined by ^{3}H-thymidine incorporation assays.

Keratinocytes, procured from neonatal foreskin after overnight trypsinization at 4°C and treatment with 0.2% EDTA, were grown in culture using a modification of the published method [Rheinwald and Green, 1975]. 3T3 cells were plated at 10^4 cells per 35-mm tissue-culture dish, and were irradiated lethally after two days with a ^{60}Co source (5,000 rad keratinocytes, in 1 ml serum-free medium, were plated on lethally irradiated 3T3 cells. Each experiment was performed on primary or secondary kerationocyte cultures obtained from different skin samples. The serum-free medium consisted of MCDB 153 medium (Sigma Chemical Co.) with additives and calcium (0.15 mM). The cells were grown to 50–60% confluence, when medium was removed and replaced with one ml of fresh medium containing either EtOH (0.1% v/v) or various doses of $1,25(OH)_2D_3$ or analog in EtOH.

^{3}H-Thymidine-Incorporation Assay

In a typical assay, cells were grown to 60–70% confluence in 24-well plates in respective media containing 5% fetal calf serum (FCS), and serum starved for 24 h, followed by treatment with various agents (in 0.1% ethanolic solution) or EtOH (vehicle) in serum-containing medium for 15 h. After the treatment, media were removed from the wells and replaced with media containing ^{3}H-thymidine (0.1 μCi) per well, and the cells were incubated for 3 h at 37°C. After this period, media was removed by aspiration and the cells were washed thoroughly (3×0.5 ml) with PBS. Then ice-cold 5% perchloric acid solution (0.5 ml) was added to each well and the cells were incubated on ice for 20 min. 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 min. 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^{-6} M of $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE, except in dose-response assays where various doses (as specified in the figures) were used. Duration of incubation for all the experiments was 16 h, except in the case of cell-cycle-analysis, which was conducted after 12 h of incubation. All the statistical analysis was performed by Student's *t*-test.

Methylene Blue Assay for Cell Viability

Briefly, the treated-plates were cooled on ice and the wells were washed twice with 0.5 ml of ice-cold PBS. Methanol (cooled to −20°C) was added to each well (0.5 ml/well) and incubated on ice for 20 min. Methanol was removed by aspiration and the plates were air-dried for 20 min. Methylene blue solution (1% solution

in 10 mM borate buffer, pH 8.5) was added to each well (0.25 ml/well) and incubated on ice for 30 min. The plates were washed four (4) times with 10 mM borate buffer, pH 8.5 and the bound dye was eluted by the addition of 1.0 ml of elution solution (ethanol:0.1 M HCl, 1:1). The intensity of color was determined spectrophotometrically at 650 nm. The viable cells were expressed as percent of control, i.e., EtOH-treated cells.

Cell-Cycle Analysis by FACS

Cell cycle analysis and the measurement of the percentage of apoptotic cells were determined by flow cytometry. Briefly, PC3 cells were cultured in 6-well plates and treated with 10^{-6} M of $1,25(OH)_2D_3$,$1,25(OH)_2D_3$-3-BE or vehicle as required for 12 h. The floating and adherent cells, collected by scraping, were washed with PBS, fixed, and permeabilized with ice-cold 70% ethanol for 30 min, followed by incubation with 50 μg/ml propidium iodide and 100 μg/ml Rnase for 30 min at 37°C in the dark. Data acquisition and analysis were performed in a flow cytometer (FACScan; BD Biosciences, Mountain View, CA), with the accompanying software (CellQuest; BD Biosciences). Ten thousand events per sample were counted. Appropriate gating was used to select the easily distinguished single cell populations. Determination of the cell cycle distribution was repeated two times. The percentage of apoptotic cells was calculated in three independent experiments.

RESULTS AND DISCUSSION

It is well-appreciated that analogs of $1,25(OH)_2D_3$ render their agonistic property by changing the conformation of VDR differently than $1,25(OH)_2D_3$ [Peleg et al., 1995; Van Den Bemd et al., 1996]. This phenomenon was verified by the determination of the three-dimensional structures of VDR, bound to $1,25(OH)_2D_3$ and its agonists [Rochel et al., 2000; Tocchini-Valentini et al., 2001]. Similarly, we showed that $1,25(OH)_2D_3$-3-BE, a mimic of $1,25(OH)_2D_3$, changed the conformation of VDR differently from $1,25(OH)_2D_3$ in manifesting its agonist action in keratinocytes [Chen et al., 1999].

Growth of a variety of normal and malignant cells are known to be modulated by $1,25(OH)_2D_3$ and its analogs [Mehta and Mehta, 2002; Holick, 2003]. Therefore, we compared the activity of $1,25(OH)_2D_3$-3-BE and $1,25(OH)_2D_3$ on the proliferation of several epithelial cells, i.e., keratinocytes, MCF-7 breast cancer, PC-3, and LNCaP prostate cancer and PZ-HPV-7 immortalized normal prostate cells.

Growth-inhibitory effect of $1,25(OH)_2D_3$ and its analogs is known to vary among cell-lines and even among lines from the same tissue. But, in general, strongest effect is observed at a 10^{-6} M concentration of the hormone or its analogs [Simbolli-Campbell et al., 1996]. Although this concentration is considered to be physiologically irrelevant, it produces optimal effect. Therefore, we chose this dose for our initial screening of various cell lines for an easier comparison.

Results of the ^{3}H-thymidine incorporation assays with 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ are shown in Figure 1. We observed that 10^{-6} M of $1,25(OH)_2D_3$-3-BE was a stronger antiproliferative agent than $1,25(OH)_2D_3$ in all the cells tested, but the activity of the analog was strongest in prostate cells (PZ-HPV-7, PC-3, and LNCaP). Furthermore, we observed that $1,25(OH)_2D_3$-3-BE was cytotoxic only to PC-3 and LNCaP cells. Upon treatment with $1,25(OH)_2D_3$-3-BE for 16 h, PC3 and LNCaP cells became apoptotic and were found detached and floating. A typical picture of LNCaP cells treated with EtOH (vehicle) or 10^{-6} M of either $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE for 16 h is shown in Figure 2, where $1,25(OH)_2D_3$-3-BE-treated cells appeared to have characteristics typical for cells undergoing apoptosis.

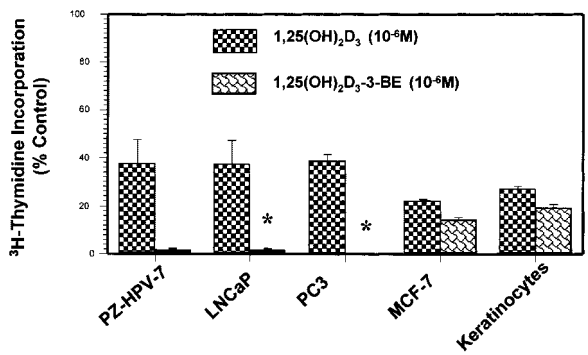

Fig. 1. ^{3}H-thymidine incorporation assays of keratinocytes, MCF-7, PC-3, LNCaP, and PZ-HPV-7 cells. Cells were treated with 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ or ethanol (EtOH) for 16 h followed by incubation with ^{3}H-thymidine and assaying for the incorporation of radioactivity in the cells. Assays were carried out with six (6) replicates and Student's *t*-test was employed for statistical analysis. *Represents cell-kill as observed under phase-contrast microscope.

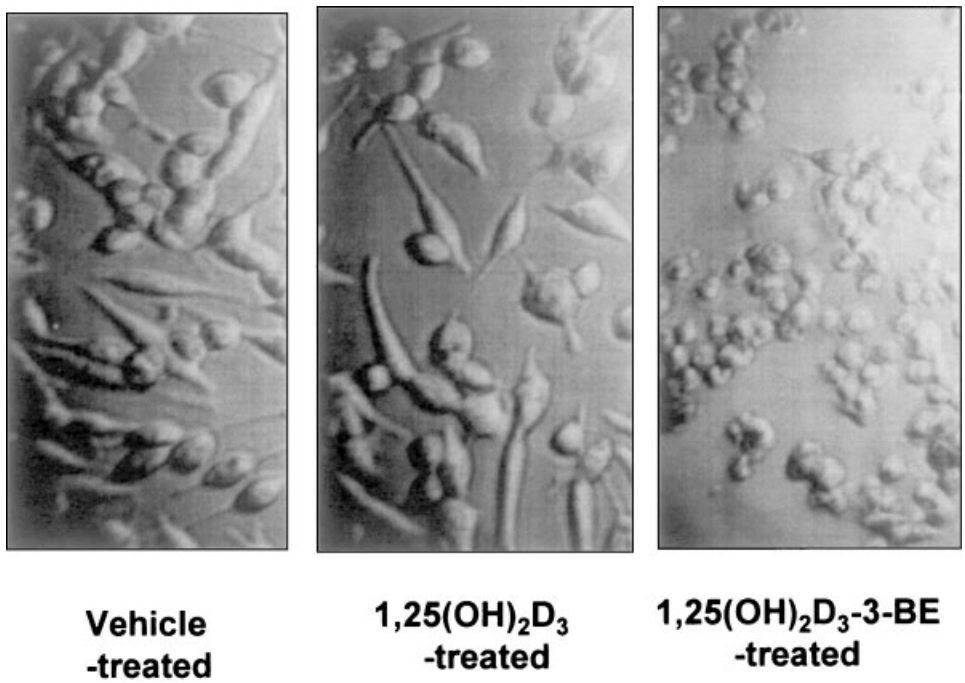

Fig. 2. Microscopic appearance of LNCaP cells treated for 16 h with EtOH (vehicle) or 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$.

The mechanism of the growth-inhibitory activity of $1,25(OH)_2D_3$ is quite complex. In most cases $1,25(OH)_2D_3$ causes the cells to be arrested in the G_0/G_1 phase; and down-regulates progression to S phase [Pols et al., 1990). For example, in human myelomonocytic cell line U937, antiproliferative effect correlated well with the expression of cyclin-dependent kinase (CDK) inhibitors, such as p21 and p27 [Liu et al., 1996]. On the other hand, in some cases, $1,25(OH)_2D_3$ reduced cell-load by inducing apoptosis or programmed cell-death. For example, Narvaez and Welsh [2001] reported that $1,25(OH)_2D_3$ induced apoptosis in MCF-7 cells. However, published reports are often conflicting. For example, Blutt et al. [2000] reported that $1,25(OH)_2D_3$ induced apoptosis in LNCaP cells, but another group failed to observe such an effect [Zhuang and Burnstein, 1998].

FACS analysis of cells, stimulated under different conditions, is a method of choice to determine the induction of apoptosis by these stimuli. Therefore, to probe the cellular toxicity of $1,25(OH)_2D_3$-3-BE in prostate cancer cells, we treated PC-3 cells with 10^{-6} M of either $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE and determined the cell cycle distribution by FACS analysis. As shown in Figure 3, while 10^{-6} M

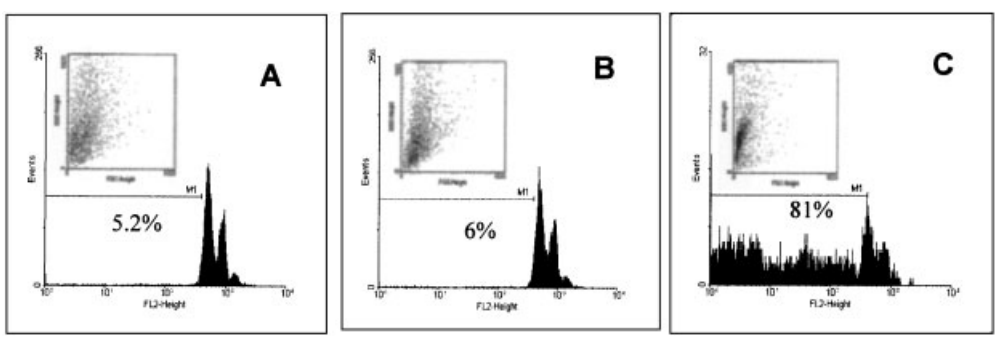

Fig. 3. FACS analysis of PC-3 cells treated with EtOH (control) or 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ for 12 h. **A**: PC-3+EtOH (control); (**B**) PC-3 + $1,25(OH)_2D_3$ (10^{-6} M); (**C**) PC-3+ $1,25(OH)_2D_3$-3-BE (10^{-6} M).

of $1,25(OH)_2D_3$ showed no significant apoptotic behavior in PC-3 cells (6% compared to 5.2% in vehicle control), an equimolar amount of $1,25(OH)_2D_3$-3-BE strongly induced apoptosis (81%).

We noted that cellular toxicity was noticeable with PC-3 and LNCaP cells only (denoted by asterisk in Fig. 1). To elaborate on this observation, we carried out ^{3}H-thymidine incorporation and methylene blue assays with LNCaP and PZ-HPV-7 cells incubated with 10^{-6} M of $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE. While the first assay was employed to measure the incorporation of ^{3}H-thymidine in the DNA of proliferating cells, the second assay was used to determine the viability of the cells after treatment with different compounds. As shown in Figures 4 and 5, $1,25(OH)_2D_3$ did not show any toxicity in LNCaP or PZ-HPV-7 cells, although it was growth-inhibitory in both the cell-lines. In contrast, $1,25(OH)_2D_3$-3-BE reduced the number of viable cells to approximately 35 and 83% in LNCaP and PZ-HPV-7 cells, while it was antiproliferative to both the cell-lines.

We observed that growth-inhibitory effect of $1,25(OH)_2D_3$-3-BE was dose-dependent in LNCaP cells, similar to $1,25(OH)_2D_3$ (Fig. 6); and the strongest effect was displayed at 10^{-6} M. But at each dose level, the effect of $1,25(OH)_2D_3$-3-BE was stronger than an equimolar amount of $1,25(OH)_2D_3$. Furthermore, the level of cellular toxicity (as determined by cell viability assay) was also dose-dependent in LNCaP cells (Fig. 7).

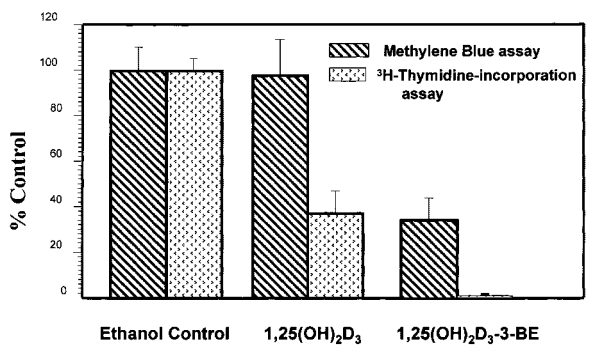

Fig. 4. Effects of 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ on growth and viability of LNCaP cells. Cells were grown to 60–70% confluence and serum-starved, followed by incubation with 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ or EtOH (vehicle) for 16 h. After this period, one set of cells was subjected to ^{3}H-thymidine incorporation assay; and the other set was subjected to methylene blue assay. Assays were carried out with six (6) replicates and Student's *t*-test was employed for statistical analysis.

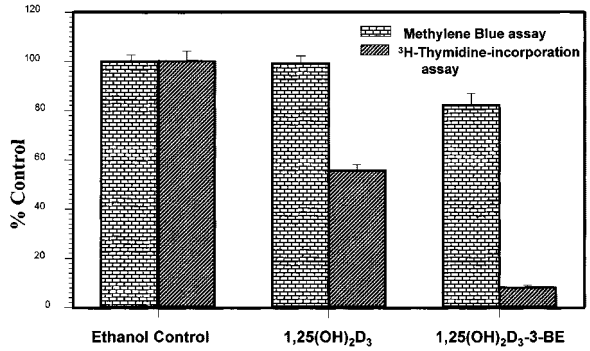

Fig. 5. Effects of 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ on growth an viability in PZ-HPV-7 cells. These assays were carried out in the same manner as described in Figure 4.

For example, number of viable cells were approximately 32 and 78% with 10^{-6} and 10^{-8} M of $1,25(OH)_2D_3$-3-BE respectively, while it was similar to the control with equivalent amounts of $1,25(OH)_2D_3$.

Growth-inhibitory effect of $1,25(OH)_2D_3$ and it analogs are dependent on concentration as well as duration of incubation. It is customary to treat cells for 48–72 h, and sometimes even longer to obtain optimal effect [Skowronski et al., 1995; Simbolli-Campbell et al., 1996; Blutt et al., 2000]. We observed that cytotoxic behavior of $1,25(OH)_2D_3$-3-B (10^{-6} M) was apparent as early as 8 h of incubation. Therefore, in all the studies (except cell-cycle-analysis) described in this report, we incubated the cells for 16 h to have optimal effects; and did not pursue the time-dependent studies any further.

$1,25(OH)_2D_3$-3-BE contains an ester bond which could potentially be hydrolyzed in a

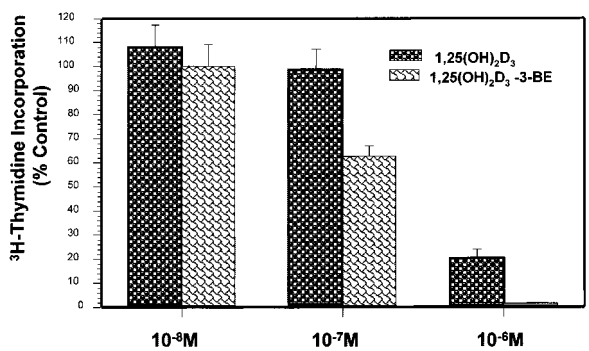

Fig. 6. Dose-response study of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ on the growth of LNCaP cells. Cells were treated with 10^{-8-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ or EtOH for 16 h, and subjected to ^{3}H-thymidine incorporation assay in the usual fashion.

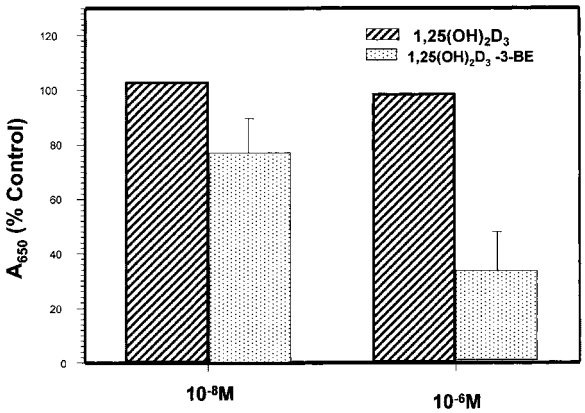

Fig. 7. Methylene blue assay of LNCaP cells treated with EtOH or 10^{-6} or 10^{-8} M of either $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$.

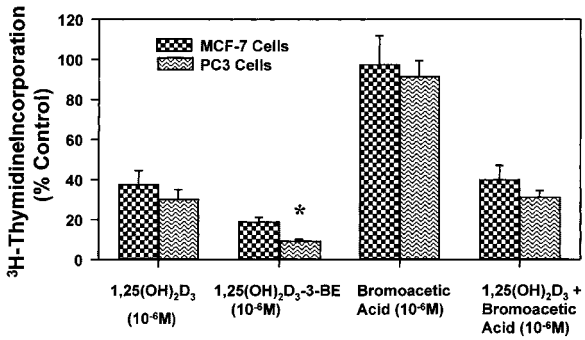

Fig. 9. Effects of 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ or bromoacetic acid or a combination of $1,25(OH)_2D_3$ and bromoacetic acid (10^{-6} M each) on the proliferation of MCF-7 and PC-3 cells. Cells were treated with EtOH or 10^{-7-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$, and subjected to ^{3}H-thymidine incorporation assay in the usual fashion. *Represents cell-kill under phase-contrast microscope.

cellular system. Such a process would produce equimolar quantities of $1,25(OH)_2D_3$ and bromoacetic acid, as shown in Figure 8. Therefore, observed antiproliferative and toxic effects could be due, at least in part, to $1,25(OH)_2D_3$ or bromoacetic acid or a combination of both. To address this issue, we incubated MCF-7 and PC-3 cells with 10^{-6} M of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ or bromoacetic acid or a combination of $1,25(OH)_2D_3$ and bromoacetic acid (10^{-6} M each), and determined their proliferation by ^{3}H-thymidine incorporation assays. Results, shown in Figure 9, demonstrated that 10^{-6} M of bromoacetic acid had no effect on the proliferation of MCF-7 and PC-3 cells. Furthermore, antiproliferative effect of a combination of $1,25(OH)_2D_3$ and bromoacetic acid (10^{-6} M each) was very similar to that of $1,25(OH)_2D_3$ (10^{-6} M) in both the cells. Therefore, these results strongly suggested that observed effects of $1,25(OH)_2D_3$-3-BE were due, at least in large part, to the un-hydrolyzed molecule.

It is also noteworthy that the cells were dosed in respective media containing FBS. FBS contains many proteins, which could potentially interact with $1,25(OH)_2D_3$-3-BE in a non-specific manner. We have also demonstrated that $1,25(OH)_2D_3$-3-BE covalently labels serum DBP [Swamy et al., 1997b]. Yet $1,25(OH)_2D_3$-3-BE displayed specific and dose-dependent effects in LNCaP and PC-3 cells. Therefore, collectively these results strongly suggested that the effect of $1,25(OH)_2D_3$-3-BE was not due to random interaction of $1,25(OH)_2D_3$-3-BE with cellular proteins.

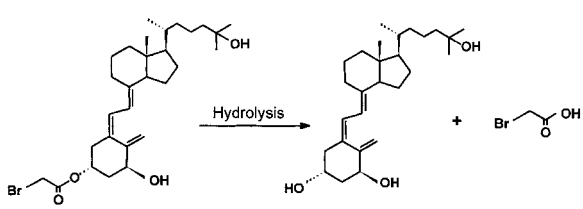

Fig. 8. Hydrolysis of the ester bond in$1,25(OH)_2D_3$-3-BE to produce equimolar quantities of $1,25(OH)_2D_3$ and bromoacetic acid.

We have shown that $1,25(OH)_2D_3$-3-BE covalently labels VDR quantitatively and in rapid fashion by a kinetic process [Ray et al., 1996; Swamy et al., 1997a]. Therefore, theoretically all of it should be attached to VDR. However, in a cellular system certain fraction of this molecule probably undergoes hydrolysis and another fraction reacts with serum DBP (present in the media). This could be the reason why micromolar dose of $1,25(OH)_2D_3$-3-BE is required for its biological activity. But, what fraction of the intact molecule is responsible for its biological activities is an open question. Currently, we are in the process of synthesizing a similar affinity-labeling analog, which is stable towards ester-hydrolysis. Such a compound might be useful in providing meaningful insight into the molecular mechanism of $1,25(OH)_2D_3$-3-BE.

Prostate cancer cell-specific effect of $1,25(OH)_2D_3$-3-BE is also an open question at this point. Simple increase in potency (by covalent labeling of VDR) cannot be the answer, because all the cells should react similarly due to the 'physical' change of the incipient VDR in all these cells by this molecule, except in the unlikely case that VDR in prostate cancer cells

is 'different' than in other cells. We speculate that $1,25(OH)_2D_3$-3-BE specifically modulates certain factor/factors directly or indirectly (via VDR) in the prostate cancer cells. Currently, we are in the process of identifying factor/factors that are involved in the prostate cancer cell-specific effect of $1,25(OH)_2D_3$-3-BE.

Tissue or cell-specific action of $1,25(OH)_2D_3$ and its mimics is not common due to the ubiquitous nature of VDR. In this report, we describe that $1,25(OH)_2D_3$-3-BE showed strongest growth-inhibitory activity in prostate cells, and cytotoxicity in malignant prostate cells only. It is well-known that $1,25(OH)_2D_3$ displays variable effects in different cell-lines, and even in cell-lines from the same tissue. For example, among human prostate cancer cell-lines, LNCaP cells are most strongly modulated, while growth-inhibition of PC-3, ALVA-31, and DU-145 cells is moderate to weak [Zhuang and Burnstein, 1998; Blutt and Weigel, 1999; Ly et al., 1999]. Such variability potentially limits the use of $1,25(OH)_2D_3$ and its analogs for the treatment and/or prevention of prostate cancer. We showed that $1,25(OH)_2D_3$-3-BE displayed similar growth-inhibitory and cytotoxic activities towards androgen sensitive LNCaP and androgen-independent PC-3 cell-lines. Therefore, these results raise the possibility that $1,25(OH)_2D_3$-3-BE or similar VDR-cross linking analogs of $1,25(OH)_2D_3$ might be considered for further development as potential candidates for prostate cancer.

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