A Vitamin D Receptor-Alkylating Derivative of 1\(\alpha\),25-Dihydroxyvitamin D\(_3\) Inhibits Growth of Human Kidney Cancer Cells and Suppresses Tumor Growth

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Abstract

1,25-Dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)] has shown strong promise as an antiproliferative agent in several malignancies, yet its therapeutic use has been limited by its toxicity leading to search for analogues with antitumor property and low toxicity. In this study, we evaluated the in vitro and in vivo properties of 1,25-dihydroxyvitamin D\(_3\)-3-bromoacetate [1,25(OH)\(_2\)D\(_3\)-3-BE], an alkylating derivative of 1,25(OH)\(_2\)D\(_3\), as a potential therapeutic agent for renal cancer. Dose response of 1,25(OH)\(_2\)D\(_3\)-3-BE in 2 kidney cancer cell lines was evaluated for its antiproliferative and apoptotic properties, and mechanisms were evaluated by Western blot and FACS analyses. Therapeutic potential of 1,25(OH)\(_2\)D\(_3\)-3-BE was assessed both by determining its stability in human serum and by evaluating its efficacy in a mouse xenograft model of human renal tumor. We observed that 1,25(OH)\(_2\)D\(_3\)-3-BE is significantly more potent than an equivalent concentration of 1,25(OH)\(_2\)D\(_3\) in inhibiting growth of A498 and Caki 1 human kidney cancer cells. 1,25(OH)\(_2\)D\(_3\)-3-BE–mediated growth inhibition was promoted through inhibition of cell-cycle progression by downregulating cyclin A and induction of apoptosis by stimulating caspase activity. Moreover, 1,25(OH)\(_2\)D\(_3\)-3-BE strongly inhibited Akt phosphorylation and phosphorylation of its downstream target, caspase-9. 1,25(OH)\(_2\)D\(_3\)-3-BE seemed to be stable in human serum. In xenograft mouse model of human renal tumor, 1,25(OH)\(_2\)D\(_3\)-3-BE was more potent at reducing tumor size than 1,25(OH)\(_2\)D\(_3\), which was accompanied by an increase in apoptosis and reduction of cyclin A staining in the tumors. These results suggest a translational potential of this compound as a therapeutic agent in renal cell carcinoma. Data from this study and extensive studies of vitamin D for the prevention of many malignancies support the potential of 1,25(OH)\(_2\)D\(_3\)-3-BE for preventing renal cancer and the development of relevant in vivo prevention models for assessing this potential, which do not exist at present.

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Introduction

Kidney cancer is among the 10 most common cancers in men and women and its rate has been increasing steadily over the past 3 decades. The American Cancer Society estimates that there were approximately 57,760 new cases of kidney cancer in the United States in the year 2009 and approximately 12,980 people died from this disease (1). Renal cell carcinoma (RCC) accounts for an estimated 90% to 95% of all kidney cancers and has been increasing at a rate of approximately 3% per year in the United States and Europe. Approximately 50% of localized RCC develops into a metastatic disease within a relatively short time frame (2). In addition, RCC characteristically produces no symptoms during its initial growth, making early diagnosis difficult, and is generally resistant to conventional chemo- and radiation therapies (2, 3). Current therapeutic options include radical nephrectomy for early-stage disease and immunotherapy for advanced and metastatic stages. Antiangiogenic agents and Raf kinase-inhibiting small molecules have also shown promise in treating RCC but are not curative (4–6). Clearly, more effective therapies and novel approaches to treatment of this disease are needed.

Numerous epidemiologic studies have shown the importance of vitamin D, dietary or otherwise, in preventing various cancers (7). In addition, the therapeutic potential of 1\(\alpha\),25-dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\)], the biologically active metabolite of vitamin D, and its analogues in cancer is well documented (8). However, the inherent calcemic toxicity of this hormone, particularly in
pharmaceutical doses, has prevented its general use as an anticancer agent (9–11). Thus, development of vitamin D analogues exhibiting potent antiproliferative activity but reduced systemic toxicity has become an active area of research (8).

We have developed novel analogues of 1,25(OH)2D3 and its prehormonal form, 25-hydroxyvitamin D3 (25-OH-D3), that specifically bind and label the ligand-pocket of the nuclear receptor for 1,25(OH)2D3 (vitamin D receptor, VDR; refs. 12, 13). Previously, we reported that 1α,25-dihydroxyvitamin D3-3-bromoacetate [1,25(OH)2D3-3-BE] and 25-hydroxyvitamin D3-3-bromoacetate (25-OH-D3-3-BE), VDR-alkylating derivatives of 1,25(OH)2D3 and 25-OH-D3, respectively, are more potent than 1,25(OH)2D3 in promoting antiproliferative effects on human cancer cell lines, including hormone-sensitive and hormone-insensitive prostate cancer cell lines (14–17). Lange et al. also reported antiproliferative and apoptotic effects of 25-OH-D3-3-BE in high-risk neuroblastoma (18).

In the present study, we compared the in vitro and in vivo growth-inhibitory properties of 1,25(OH)2D3-3-BE with 1,25(OH)2D3 in human renal cancer cells and examined potential molecular mechanisms underlying its activities. We observed that 1,25(OH)2D3-3-BE is more potent than 1,25(OH)2D3 in inhibiting the growth of A498 and Caki 1 renal cancer cells. Mechanistically, 1,25(OH)2D3-3-BE-mediated growth inhibition of renal cancer cells was associated with an increase in apoptosis, arrest in the G2/M checkpoint in the cell cycle, and inhibition of Akt phosphorylation. In nude mice, 1,25(OH)2D3-3-BE was more potent at reducing xenografted tumor size than 1,25(OH)2D3, which was accompanied by an increase in apoptosis and reduction of cyclin A staining in the tumors.

Materials and Methods

Materials

1,25(OH)2D3-3-BE was synthesized according to our published procedure (19). 1α,25-Dihydroxyvitamin D3-3-[1,4C]bromoacetate [14C-1,25(OH)2D3-3-BE, specific activity 14.3 mCi/mmol] was synthesized by replacing bromoacetic acid in the synthetic scheme with [1-14C]bromoacetic acid (sp. activity 14.3 mCi/mmol; DuPont, New England Nuclear). Its radiochemical purity was ascertained by co-HPLC analysis with a standard of 1,25(OH)2D3-3-BE, which was a generous gift from Dr. Milan Uskokovic (Hoffman-LaRoche). Concentrations of 1,25(OH)2D3 and 1,25(OH)2D3-3-BE were determined spectrophotometrically using an extinction coefficient of 18,400 at 265 nm. Purity of the compounds was determined by HPLC analysis (normal and reverse phases). LY294002 was from Cell Signaling Technology. A498 (HTB-44) and Caki 1 (HTB-46) cell lines were purchased from ATCC and maintained in DMEM with 10% FBS. Three- to 4-week-old athymic male mice (average weight 20 g) were purchased from Taconic Farms and maintained in an AAALAC-approved animal care facility at Boston University School of Medicine.

Cellular proliferation assay

Cellular proliferation was measured using the TACS MTT Cell Proliferation kit according to the manufacturer’s instructions (Trevigen). Briefly, A498 and Caki 1 cells were plated in 96-well plates at 1,000 cells per well. Sixteen hours later, cells were treated with 1,25(OH)2D3-3-BE, 1,25(OH)2D3 or ethanol (vehicle) control in media containing 5% FBS. The medium containing compounds was replenished every 2 days. After 7 days, MTT solution was added to each well, and the plates were incubated at 37°C for 3 hours, followed by the addition of detergent reagent. The plates were incubated at 25°C for 15 hours and absorbance at 570 nm was measured on a microplate reader (Spectramax 190 Plate Reader; Molecular Devices).

Caspase activity assay

Caspase activity was determined using the Apo-ONE Homogeneous Caspase-3/7 assay according to the manufacturer’s instructions (Promega). Caspase-3/7 activity was determined following treatment of Caki 1 cells for 6 hours with 1,25(OH)2D3, 1,25(OH)2D3-3-BE, or ethanol (vehicle) control. Fluorescence released following cleavage of the profluorescent substrate, Z-DEVD-110, was measured at the emission maximum of 521 nm. The amount of fluorescent product generated is representative of the amount of active caspase-3/7 in the sample.

Cell cycle analysis and sub-G0/G1 DNA content

A498 cells were plated at 5 × 104 cells in 10-cm tissue culture dishes. Sixteen hours later, the cells were treated with 1,25(OH)2D3-3-BE, 1,25(OH)2D3, or ethanol control for 6 hours. The cells were trypsinized, collected, and centrifuged at 1,500 rpm for 5 minutes. They were resuspended in 1.5 mL saponin/PI solution [0.3% saponin (w/v), 2.5% PI (w/v), 0.1 mmol/L EDTA, 10 μg/mL RNase in PBS] and incubated overnight in the dark. FACS analysis was carried out using a Beckman Coulter FC500 flow cytometer. ModFit LT software (Verity Software House) was used for analysis.

Western blot analysis

A498 and Caki 1 cells were plated at 3 × 105 cells in 6-cm tissue culture dishes. Sixteen hours later, the cells were treated with 1,25(OH)2D3-3-BE, 1,25(OH)2D3, or ethanol (vehicle control). At the indicated times, the cells were washed with PBS, scraped in PBS, and collected by centrifugation. Total cellular extracts were prepared by resuspending the cell pellets in RIPA buffer (150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/M Tris, pH 7.5) containing 50 mmol/L NaF, 1 mmol/L sodium vanadate (Na3VO4) and protease inhibitors (Protease Inhibitor Cocktail; Sigma-Aldrich). Following 10-minute incubation of the samples on ice, the extracts were cleared by microcentrifugation for 10 minutes at 14,000 rpm, supernatants were transferred to new tubes, and protein concentration of each extract was determined by the Bradford assay. Samples were separated on 4% to 12% MES NuPAGE gels (Invitrogen) and transferred to
PVDF membrane. Signals were detected by enhanced chemiluminescence (Perkin Elmer) and autoradiography. The antibodies used were anti-Akt and anti-phospho-Akt (Ser 473; Cell Signaling, #9272 and #9271, respectively), anti-phospho-caspase-9 (Ser 196; Santa Cruz; #11755), anti-cyclin A (Neomarkers; Rb-1548), and anti-β-actin (Sigma; #A5441).

**Serum stability of 1,25(OH)$_2$D$_3$-3-BE**

Pooled human serum (1 mL) was spiked with $^{14}$C-1,25(OH)$_2$D$_3$-3-BE (100,000 cpm) for 1 hour at 37°C followed by extraction with 5 × 1 mL of ethyl acetate. Combined organic extract was dried under argon, and the residue was redissolved in a small volume of 5% H$_2$O/MeOH and analyzed in an Agilent 1100 Series HPLC system (Thermo-Fisher), connected to a Packard Flow Scintillation Analyzer (model no. 150TR), using 5% H$_2$O/MeOH as the mobile phase, flow rate 1.5 mL/min, detection 265 nm (for nonradioactive materials), Agilent C18 analytical column (Thermo-Fisher). Prior to the analysis of the radioactive sample, a mixture of standard samples of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$-3-BE was analyzed under the same conditions as that in HPLC analysis.

**Xenograft tumor growth in athymic mice**

Caki 1 cells were grown, trypsinized, and resuspended in PBS to obtain a concentration of 5 × 10$^6$ cells/100 μL. Cell suspensions (100-μL aliquots) were injected subcutaneously in the flanks of athymic mice. When tumors grew to approximately 100 mm$^3$, the animals were separated into 6 animals per group and were treated with 1,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$-3-BE, 1,25(OH)$_2$D$_3$ (dissolved in 5% dimethylacetamide in sesame oil, 0.75 μg/kg of body weight/100 μL), or vehicle by intraperitoneal injection every third day. Tumor size and body weights were measured on days of injection. Treatments stopped once the control group tumors reached an average volume of 1.5 cm$^3$ and then animals were killed. Tumors were excised and stored in 10% neutral buffered formalin, and blood samples were collected by cardiac puncture. Statistical analysis of tumor size was carried out by Student's t test. Serum calcium of the treated animals was measured according to manufacturer's instructions (Quantichrom Calcium Assay Kit, #DICA-500; BioAssay Systems).

**Histochemistry**

Mouse tumors were fixed in 10% neutral buffered formalin for 48 hours before tissue processing into paraffin wax. Five-micrometer sections were cut and mounted onto positively charged slides. Hematoxylin and eosin (H&E) staining was carried out using standard methods. Briefly, sections were deparaffinized with xylene, rinsed through graded alcohols, and hydrated to water. The nuclei were stained for 5 minutes in Harris hematoxylin (Anatech) and differentiated in acid alcohol (1% HCl in 70% alcohol by volume) and 1% ammonium hydroxide. The nonnuclear elements were stained with alcoholic eosin (Anatech) for 3 dips and dehydrated through graded alcohols to xylene. The sections were covered with cover slips, using Cytoseal 60 synthetic resin (Richard Allan via Thermo Fisher).

**Immunohistochemistry**

Antibodies for cyclin A (LabVision via Thermo Fisher) were optimized for immunohistochemistry on the Ventana NexES autostainer (Ventana Medical Systems) at an operating temperature of 37°C. Five-micrometer fresh-cut paraffin sections were deparaffinized in xylene, rinsed in graded alcohols, and hydrated to water. Antigen retrieval was carried out in a Decloaker chamber for 5 minutes at 125°C (22 psi). The retrieval solution was BORG, pH 9.5. Primary antibody for cyclin A was used at 1:100 and incubated for 30 minutes. A Ventana I-VIEW detection kit was modified to detect only rabbit antibodies by substituting a biotinylated goat anti-rabbit secondary (Jackson Immunoresearch) diluted 1:50 in PBS, pH 7.6. Sections were counterstained in Mayer's hematoxilin for 2 minutes, dehydrated in alcohols, cleared in xylene, and coverglass mounted as for histochemistry.

**Pathologic analysis**

H&E-stained and anti-cyclin A–stained tissue sections of subcutaneous tumors were examined by a single pathologist (M.S.L.) blinded as to the treatment group. On H&E sections, the number of apoptotic bodies in the tumors per 10 random high-power (400×) fields was recorded for each animal. For cyclin A analysis, the percentage of brown cyclin A–positive tumor nuclei was assessed for each tumor, counting 500 nuclei in multiple random fields.

**Results**

1,25(OH)$_2$D$_3$-3-BE is more potent than 1,25(OH)$_2$D$_3$ in inhibiting the growth of renal carcinoma cells

In this study, we examined the effect of 1,25(OH)$_2$D$_3$-3-BE on the growth of the human renal cancer cell lines A498 and Caki 1. Cells were treated with 1,25(OH)$_2$D$_3$-3-BE or 1,25(OH)$_2$D$_3$ and cellular proliferation was quantitated by the MTT assay. In both A498 and Caki 1 cells, treatment with 10$^{-6}$ mol/L of 1,25(OH)$_2$D$_3$-3-BE almost completely inhibited cellular proliferation, whereas an equivalent amount of 1,25(OH)$_2$D$_3$ inhibited growth by approximately 10% (Fig. 1). Caki 1 cells were more sensitive to 1,25(OH)$_2$D$_3$-3-BE than were A498 cells. Approximately 90% growth inhibition was observed with 10$^{-7}$ mol/L of 1,25(OH)$_2$D$_3$-3-BE in Caki 1 cells, whereas approximately 30% growth inhibition was observed in A498 cells (Fig. 1). These results demonstrate that 1,25(OH)$_2$D$_3$-3-BE elicits stronger antiproliferative effects in A498 and Caki 1 cells than 1,25(OH)$_2$D$_3$ on an equimolar basis.

Under microscopic visualization, we noted distinct morphologic changes in the appearance of both A498 and Caki 1 cells in response to 1,25(OH)$_2$D$_3$-3-BE treatment. As shown in Figure 1B, after 6 hours of treatment with 1,25(OH)$_2$D$_3$-3-BE, both A498 and Caki 1 cells displayed cell rounding and began detaching from the plates. Interestingly, cells treated with 1,25(OH)$_2$D$_3$ did not display
these characteristics and exhibited morphologic features similar to vehicle-treated control cells.

**1,25(OH)2D3-3-BE promotes G2/M arrest of A498 cells**

The cellular mechanism(s) leading to growth inhibition by 1,25(OH)2D3 is complex. In prostate cancer cells, 1,25(OH)2D3 causes cells to arrest in the G0/G1 phase of the cell cycle (20). This effect is thought to be mediated by increased expression of the cyclin-dependent kinase (CDK) inhibitors p21 and p27 and other cell-cycle regulators (21, 22). To examine the effect of 1,25(OH)2D3-3-BE on cell-cycle progression in A498 cells, we measured cell-cycle distribution by flow cytometry of propidium iodide (PI)-stained cells following 6 hours of exposure to 1,25(OH)2D3-3-BE, 1,25(OH)2D3, or ethanol (vehicle control). As shown in Figure 2A, the cell-cycle distributions were similar in control and in 1,25(OH)2D3-treated cells. However, cells, treated with 1,25(OH)2D3-3-BE showed an approximately 2-fold increase in the relative proportion of cells in G2/M compared with control and 1,25(OH)2D3-treated cells. In addition, a population of cells with a subdiploid DNA content appeared, suggesting that 1,25(OH)2D3-3-BE may activate a G2/M checkpoint arrest in renal cancer cells, preventing progression through the cell cycle.

**1,25(OH)2D3-3-BE reduces the level of cyclin A in A498 and Caki 1 cells**

Cyclins control progression through the cell cycle via their association with CDKs. Cyclin A controls the transition from G2 to mitosis and its expression has been shown to have predictive value in the clinical stages of renal cancer (23, 24). Because of our observation that 1,25(OH)2D3-3-BE arrests cells in the G2/M checkpoint, and the importance of cyclin A in renal cancer, we investigated cyclin A expression in A498 and Caki 1 cells treated with
either 1,25(OH)_{2}D_{3} or 1,25(OH)_{2}D_{3}-3-BE. We observed that 6-hour treatment of Caki 1 and A498 cells with 10^{-6} mol/L of 1,25(OH)_{2}D_{3}-3-BE strongly reduced cyclin A, while the same concentration of 1,25(OH)_{2}D_{3} failed to do so (Fig. 2B), indicating that 1,25(OH)_{2}D_{3}-3-BE may cause arrest at the G2/M checkpoint in these cells through downregulation of cyclin A.

1,25(OH)_{2}D_{3}-3-BE treatment induces apoptosis in Caki 1 cells

Cellular growth inhibition mediated by 1,25(OH)_{2}D_{3} correlates with increased apoptosis in some studies. For example, it is reported that 1,25(OH)_{2}D_{3} induces apoptosis in LNCaP prostate cancer and MCF-7 breast cancer cells (25, 26), but this result is not universal (20). Previously, we reported that 1,25(OH)_{2}D_{3}-3-BE induces apoptosis in PC-3 prostate cancer cells (15, 16). Thus, we investigated the role of apoptosis in 1,25(OH)_{2}D_{3}-3-BE–mediated growth inhibition of renal cancer cells.

We observed rounding and sloughing of cells treated with 1,25(OH)_{2}D_{3}-3-BE but not with 1,25(OH)_{2}D_{3} or vehicle control in both Caki 1 and A498 cell lines (Fig. 1B). To determine whether the striking morphologic changes in kidney cancer cells in response to 1,25(OH)_{2}D_{3}-3-BE treatment are related to induction of apoptosis, we carried out flow cytometric analysis of nuclear DNA
content following exposure to 1,25(OH)\(_2\)D\(_3\)-3-BE or 1,25(OH)\(_2\)D\(_3\) in Caki 1 cells. As shown in Figure 3A, the sub-G\(_0\)/G\(_1\) (hypodiploid) fraction, indicative of apoptotic cells, was equivalent between control and 1,25(OH)\(_2\)D\(_3\)-treated cells (8%–11%). However, the 1,25(OH)\(_2\)D\(_3\)-3-BE–treated cells showed a large increase in this sub-G\(_0\)/G\(_1\) population (74%).

Caspases are a family of proteases that play an essential role in apoptotic cell death, and caspase activation is considered a hallmark of apoptosis. To examine the role of 1,25(OH)\(_2\)D\(_3\)-3-BE in caspase activation, we carried out a caspase activity assay on Caki 1 cells following treatment with 1,25(OH)\(_2\)D\(_3\) and 1,25(OH)\(_2\)D\(_3\)-3-BE. This assay detects activation of caspase-3 and -7 through cleavage of a fluorescent substrate specific for caspase-3 and -7. As seen in Table 1, no caspase activity was observed in cells treated with ethanol control or 1,25(OH)\(_2\)D\(_3\). However, strong activation of caspase-3 and -7 activity was observed in cells treated with 1,25(OH)\(_2\)D\(_3\)-3-BE. Taken together, the results of sub-G\(_0\)/G\(_1\) DNA analysis and the caspase activation assay show the ability of 1,25(OH)\(_2\)D\(_3\)-3-BE to stimulate apoptosis in renal cancer cells.

1,25(OH)\(_2\)D\(_3\)-3-BE inhibits Akt phosphorylation in A498 cells

To investigate the molecular mechanism of 1,25(OH)\(_2\)D\(_3\)-3-BE–induced apoptosis in renal cancer cells, we examined the activation status of the prosurvival kinase Akt in A498 cells. Akt is activated by its phosphorylation at threonine 308 and serine 473, events which promote cell survival and proliferation (27). Therefore, we analyzed the activation status of Akt by immunoblot analysis with an antibody specifically recognizing phosphorylated Akt (p-Akt). The blot was stripped and reprobed for total Akt as a loading control. The results are representative of 2 independent experiments. C, 1,25(OH)\(_2\)D\(_3\)-3-BE inhibits Akt-mediated phosphorylation of caspase-9. Caki 1 cells were incubated with 5 × 10\(^{-7}\) mol/L of 1,25(OH)\(_2\)D\(_3\), 1,25(OH)\(_2\)D\(_3\)-3-BE, the PI3K/Akt inhibitor LY294002 (10 \(\mu\)mol/L), or ethanol control for 6 hours and Western blot analysis was used to assess the levels of phosphorylated Akt (p-Akt) and caspase-9 (p-caspase 9). The blot was stripped and reprobed for \(\beta\)-actin as a loading control. The results are representative of 3 independent experiments.
Table 1. 1,25(OH)₂D₃-3-BE stimulates caspase-3/7 activity in Caki 1 cells

<table>
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NOTE: Caspase-3/7 activity was determined following treatment of Caki 1 cells for 6 hours with 1,25(OH)₂D₃, 1,25(OH)₂D₃-3-BE, or ethanol (vehicle) control. Fluorescence released following cleavage of the profluorescent substrate Z-DEVD-110 was measured at the emission maximum of 521 nm. The amount of fluorescent product generated is representative of the amount of active caspase-3/7 in the sample.

Abbreviation: SE, standard error.

reduced the level of phosphorylated Akt in both cell lines. An equimolar concentration of 1,25(OH)₂D₃ also reduced Akt phosphorylation but to a much lower extent than did 1,25(OH)₂D₃-3-BE. These results suggest that the apoptotic function of 1,25(OH)₂D₃-3-BE in renal cancer cells may be mediated, at least partially, by inhibition of signaling through the Akt pathway.

Caspase-9 is a downstream target of Akt. Activated (phosphorylated) Akt phosphorylates caspase-9 on serine 196 and inhibits its protease activity leading to cell survival. Thus, a potential molecular mechanism whereby 1,25(OH)₂D₃-3-BE promotes apoptosis centers on the ability of 1,25(OH)₂D₃-3-BE to inhibit Akt activation resulting in increased caspase-9 activity. To address this hypothesis, we examined caspase-9 phosphorylation in Caki 1 cells following treatment with 1,25(OH)₂D₃-3-BE and 1,25(OH)₂D₃. As shown in Figure 3C, 1,25(OH)₂D₃-3-BE, but not 1,25(OH)₂D₃, inhibited phosphorylation of caspase-9. As a control, we used the phosphatidylinositol-3-kinase (PI3K)/Akt inhibitor LY294002 to confirm that the inhibition of Akt activity leads to decreased phosphorylation of caspase-9. These results further implicate Akt and its downstream target, caspase-9, as targets for the molecular mechanism whereby 1,25(OH)₂D₃-3-BE promotes apoptosis in renal cancer cells.

1,25(OH)₂D₃-3-BE is stable in human serum

HPLC-profile of ¹⁴C-1,25(OH)₂D₃-3-BE, incubated in human serum for 60 minutes at 37°C shows a single peak (Fig. 4B) that matches the peak for a standard sample of ¹⁴C-1,25(OH)₂D₃-3-BE (Fig. 4A), indicating that ¹⁴C-1,25(OH)₂D₃-3-BE is stable in human serum at 37°C for at least 1 hour.

1,25(OH)₂D₃-3-BE inhibits tumor growth in a mouse xenograft model

The effect of 1,25(OH)₂D₃-3-BE on the growth of renal cell tumors was evaluated in xenografts in nude mice. Caki 1 cells were injected subcutaneously in athymic nude mice and allowed to grow until the tumors reached approximately 100 mm³ in size at which time 1,25(OH)₂D₃-3-BE, 1,25(OH)₂D₃, or vehicle control was administered. In comparison with 1,25(OH)₂D₃-3-BE and 1,25(OH)₂D₃ treatment, the vehicle-treated control animals generated tumors that grew rapidly throughout the time course. In contrast, the tumors in the 1,25(OH)₂D₃-3-BE–treated group showed a significant reduction in size compared with the tumors of control animals and 1,25(OH)₂D₃-3-BE was more effective than 1,25(OH)₂D₃ in inhibiting tumor growth (Fig. 5A). To examine potential toxic effects of 1,25(OH)₂D₃-3-BE treatment, the body weights of the mice were determined each time compounds were administered. As shown in Fig. 5B, we did not observe a difference in body weights between any of the treatment groups. Importantly, serum calcium values of the 1,25(OH)₂D₃-3-BE and 1,25(OH)₂D₃-3-BE–treated animals were not significantly different from the vehicle control (Fig. 5C), denoting lack of toxicity. Collectively, these results demonstrate that 1,25(OH)₂D₃-3-BE is an effective agent at reducing renal cancer xenografts and seems to be well tolerated at this dose and time course.

1,25(OH)₂D₃-3-BE reduces cyclin A levels and increases apoptosis in tumor samples

We observed significant inhibition of cyclin A levels in our cell culture models of 1,25(OH)₂D₃-3-BE action. Therefore, we examined cyclin A staining in tumors from mice treated with 1,25(OH)₂D₃-3-BE, 1,25(OH)₂D₃, and vehicle (control). Immunohistochemical analysis of cyclin A in the xenografts showed significant reduction in the percentage of cells having nuclear cyclin A staining with both 1,25(OH)₂D₃ and 1,25(OH)₂D₃-3-BE (Fig. 6A). Importantly, the reduction in cyclin A was more pronounced in tumors derived from 1,25(OH)₂D₃-3-BE–treated animals (Fig. 6C).

Because we observed potent stimulation of apoptosis by 1,25(OH)₂D₃-3-BE in vitro, we examined the presence of apoptotic bodies, as an indication of apoptosis, in the xenografts. The number of apoptotic bodies per high-power field was increased in tumors from the 1,25(OH)₂D₃-3-BE–treated animals, suggesting that 1,25(OH)₂D₃-3-BE stimulated apoptosis in vivo (Fig. 6B and C). However, 1,25(OH)₂D₃ did not significantly increase apoptosis in the xenografts. These findings are in concordance with our observations that 1,25(OH)₂D₃-3-BE is a more potent inducer of apoptosis in renal cancer cells in vitro than 1,25(OH)₂D₃.

Discussion

There is a paucity of information about the effect of 1,25(OH)₂D₃ and its analogues in renal cancer. Nagakura et al. demonstrated that 1,25(OH)₂D₃ and some of its metabolites inhibited the growth of renal cancer cell line KU-2 (28). In addition, Fuzioka et al. showed that 1,25(OH)₂D₃ inhibited the growth of murine Renca renal cancer
cell line–induced tumor in a mouse model (29). These results suggest the potential utility of 1,25(OH)2D3 and its analogues in treating renal cancer.

We observed that 1,25(OH)2D3-3-BE is a significantly stronger antiproliferative agent than equimolar amounts of 1,25(OH)2D3 both in vitro (Fig. 1) and in a mouse xenograft tumor model (Fig. 5). Greater efficacy of 1,25(OH)2D3-3-BE than 1,25(OH)2D3 can potentially be explained by its proposed ability to titrate and engage all VDR molecules because of the kinetic nature of the alkylation process. This is an important consideration in cases in which VDR level is low. For example, Trydal et al. determined VDR level in 23 primary renal cell carcinomas and compared these levels with autologous normal kidney tissue. They reported that VDR levels for the renal cell carcinomas were approximately 3 times lower than autologous normal kidney tissue (30). We evaluated VDR levels in Caki 1 and A498 cells, treated with 1,25(OH)2D3, 1,25(OH)2D3-3-BE, or vehicle, and observed comparable levels of VDR by immunoblot analysis, suggesting that changes in VDR levels do not reflect response to 1,25(OH)2D3-3-BE (data not shown).

In Caki 1 cells, we observed that 1,25(OH)2D3-3-BE induces apoptosis, in addition to cell-cycle arrest, as evidenced by sub-G0/G1 DNA analysis and arrest at the G2/M checkpoint (Figs. 2 and 3). 1,25(OH)2D3-3-BE also strongly stimulated caspase-3/7 activity, a hallmark of apoptosis (Table 1). The induction of apoptosis by 1,25(OH)2D3 has been shown to involve upregulation...
of proapoptotic Bax and Bcl-xl, Bcl-2 family proteins that regulate the intrinsic pathway for apoptotic induction (25, 26). However, in A498 cells, 1,25(OH)2D3-3-BE, as well as 1,25(OH)2D3, failed to activate these proteins (data not shown), suggesting that activation of caspases by 1,25(OH)2D3-3-BE in kidney cancer cells may follow a different pathway.

Akt is a serine/threonine kinase that is activated by many signals in a PI3K-dependent manner (31, 32). Akt is involved in a variety of normal and tumorigenic functions such as cell proliferation, growth, and survival. Hara et al. screened 45 tumor samples from patients with renal cell carcinoma and reported that phosphorylated Akt expression increased significantly in comparison with associated normal kidney tissue and that an Akt inhibitor induced apoptosis in KU-19-20 and Caki 2 cells that have high Akt activity (33). We observed that 1,25(OH)2D3-3-BE strongly inhibited Akt phosphorylation in A498 and Caki 1 cells (Fig. 3B), indicating that the ability of 1,25(OH)2D3-3-BE to inhibit Akt activation may be critical in the molecular mechanism of its action.

Caspase-9 is a downstream effector of Akt activity. As presented in Figure 3B, we observed complete inhibition of caspase-9 phosphorylation following 1,25(OH)2D3-3-BE treatment of Caki 1 cells. Interestingly, 1,25(OH)2D3 did not inhibit caspase-9 phosphorylation, potentially revealing a key mechanism explaining the observed differences in the ability of 1,25(OH)2D3-3-BE and 1,25(OH)2D3 to promote apoptosis in renal cancer cells.

The stability of a drug in serum is a key pharmacokinetic property. Serum stability is particularly important for 1,25(OH)2D3-3-BE, because it contains an ester bond that may be prone to hydrolysis by esterases. Therefore, we determined the stability of 1,25(OH)2D3-3-BE in human serum. HPLC profile of an organic extract of a serum sample, spiked with 14C-1,25(OH)2D3-3-BE, showed the intact peak of 14C-1,25(OH)2D3-3-BE after 1-hour incubation at 37°C (Fig. 4). This result attests to...
Fig. 6. 1,25(OH)$_2$D$_3$-3-BE stimulates apoptosis and reduces cyclin A levels in renal cancer xenografts. A, immunohistochemical analysis of cyclin A levels in xenografts. The tumors derived from control, 1,25(OH)$_2$D$_3$-, and 1,25(OH)$_2$D$_3$-3-BE–treated mice were examined for cyclin A levels. Arrows indicate positive nuclear staining for cyclin A. B, apoptotic bodies are increased in tumors from 1,25(OH)$_2$D$_3$-3-BE–treated mice. Circles indicate representative apoptotic bodies. C, quantification of cyclin A staining (left) and apoptotic bodies (right). Positive nuclear staining for cyclin A and the number of apoptotic bodies in control tumors (Con), tumors derived from 1,25(OH)$_2$D$_3$–treated mice (D), and tumors derived from 1,25(OH)$_2$D$_3$–3-BE–treated mice (BE) were counted as described in the Materials and Methods section. For cyclin A statistical analysis (Student’s t test), *P < 0.005; **P < 0.0005. For the number of apoptotic bodies, *P < 0.02.
the stability of 1,25(OH)2D3-3-BE in serum and enhances its potential as a therapeutic agent.

To evaluate the potential of 1,25(OH)2D3-3-BE in renal cancer, we carried out an in vivo study with an athymic mouse model of human renal cancer. In this study, we observed that tumors in vehicle-treated (control) animals grew rapidly throughout the time course. Significantly, 1,25(OH)2D3-3-BE, but not 1,25(OH)2D3, inhibited tumor growth (Fig. 5A), reflecting the in vivo growth-inhibitory property of 1,25(OH)2D3-3-BE in kidney cancer cells. In addition, higher efficacy of 1,25(OH)2D3-3-BE in inhibiting tumor growth compared with 1,25(OH)2D3 was reflected by decreased cyclin A nuclear staining and increased apoptosis in the tumors (Figs. 6A–C). It is noteworthy that the molecular weights of 1,25(OH)2D3-3-BE and 1,25(OH)2D3 are 537.80 and 416.65 kD, respectively. Therefore, if we consider equimolar concentrations of these compounds, 1,25(OH)2D3-3-BE is actually approximately 1.3-fold more active than 1,25(OH)2D3.

1,25(OH)2D3-3-BE did not show significant toxicity, as reflected in the gross body weights of the animals throughout the study (Fig. 5B). As indicated in Fig. 5B, 1,25(OH)2D3-3-BE apparently caused some weight gain. But upon statistical analysis, body weights of 1,25(OH)2D3-treated animals were not significantly different from other groups [vehicle control and 1,25(OH)2D3-3-BE]. Furthermore, serum calcium values were not significantly different among the groups (Fig. 5C).

We have demonstrated that 1,25(OH)2D3-3-BE covalently attaches to the ligand-binding pocket of VDR (13), thus possibly making it less prone to catabolic degradation and higher activation of VDR. It can be argued that such a process may lead to “apparent higher dose of 1,25(OH)2D3” and enhance toxicity. But increasing the effective dose of 1,25(OH)2D3 by covalent labeling also means that we will require less of 1,25(OH)2D3-3-BE to bring about significant effect. We chose a dose of 0.75 μg/kg for both 1,25(OH)2D3 and 1,25(OH)2D3-3-BE at which level none of them showed any toxicity (Fig. 5B and C).

In summary, the results presented herein show that 1,25(OH)2D3-3-BE strongly suppresses growth of kidney cancer cells in vitro and tumor growth in vivo. These studies suggest further preclinical investigations and continued mechanistic investigations of 1,25(OH)2D3-3-BE in inhibiting renal cancer tumorigenesis are warranted to evaluate its translational potential as a therapeutic agent in renal cell carcinoma. Considered together with extensive data on vitamin D in various cancer prevention settings, these results also have important implications for renal cell cancer prevention. There are, however, no preclinical in vivo models, for example, genetically engineered models, for prevention research in this setting and so such models should be developed for studying the vitamin D analogue reported here as well as for studying other potentially effective preventive agents. Such prevention study would be especially relevant for people at a high risk of renal cell cancer (34–36).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


14. Chen ML, Ray S, Swamy N, Holick MF, Ray R. Mechanistic studies to evaluate the enhanced antiproliferation of human keratinocytes by 1α,25-dihydroxyvitamin D3-3-bromoacetate, a covalent modifier of...
vitamin D receptor, compared to 1α,25-dihydroxyvitamin D3. Arch Biochem Biophys 1999;370:34–44.
22. Moffatt KA, Johannes WU, Hedlund TE, Miller GJ. Growth inhibitory effects of 1α,25-dihydroxyvitamin D(3) are mediated by increased levels of p21 in the prostatic carcinoma cell line ALVA-31. Cancer Res 2001;61:7122–9.
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