Growth Suppression of Ovarian Cancer Xenografts in Nude Mice by Vitamin D Analogue EB1089

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ABSTRACT

Purpose: The poor response of advanced epithelial ovarian cancer to current treatments necessitates the development of alternative therapeutic strategies. Inhibition of cancer growth by 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] compounds represents an innovative approach for cancer therapy. The current study evaluated the therapeutic potential of a synthetic 1,25(OH)$_2$D$_3$ analogue EB1089 in the treatment of ovarian cancer.

Experimental Design: The response of human ovarian cancer cells to 1,25(OH)$_2$D$_3$ and EB1089 were first compared in cell growth, gene transcription, and apoptotic assays. Then, nude mice bearing OVCAR3 tumor xenografts were treated with EB1089 at different dosages, and tumor volumes were monitored. The effect of EB1089 and 1,25(OH)$_2$D$_3$ on the level of serum calcium was also examined. After the treatment, tumors were excised and processed for histologic examination, Ki-67 staining, and tissue terminal deoxynucleotide transferase–mediated dUTP nick end labeling (TUNEL) assays to evaluate the morphologic, proliferative, and apoptotic changes induced by EB1089, respectively.

Results: The study shows that EB1089 suppresses the in vitro growth of ovarian cancer cells and transcriptionally activates the GADD45 reporter gene more effectively than 1,25(OH)$_2$D$_3$. Clinically more importantly, EB1089 suppresses the growth of OVCAR3 tumor xenografts in nude mice without inducing hypercalcemia. Ki-67 staining and tissue TUNEL assays showed that both inhibition of cell proliferation and induction of apoptosis contribute to the EB1089-induced tumor suppression in vivo.

Conclusions: This study is the first demonstration that ovarian cancer responds positively in vivo to treatment with a 1,25(OH)$_2$D$_3$ compound and thus supports continued development of 1,25(OH)$_2$D$_3$ analogues for possible use as an alternative or complementary therapy for human ovarian cancer.

INTRODUCTION

Ovarian cancer is the leading cause of death among gynecologic malignancies, which results in more deaths than all other gynecologic cancers combined (1). In contrast to declining death rates for cervical and uterus cancers, the annual report of ovarian cancer mortality has risen by 250% since 1930 (2). The prognosis is generally poor for ovarian cancer patients of whom only ~32% survive for >5 years. The dismal response of advanced ovarian cancer to current treatments necessitates the development of alternative therapies to curb this deadly disease.

The active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$], has been shown to regulate differentiation and proliferation of multiple human cancer cell lines and thus is a potential therapeutic agent for cancers. A suppressive role for 1,25(OH)$_2$D$_3$ in ovarian cancer has been speculated based on the inverse correlation between ovarian cancer incidence and mortality rates and sunlight exposure (3, 4). In addition, a case-control study in Mexico reported an inverse association between dietary vitamin D content and risk for ovarian cancer (5).

Consistent with these epidemiologic analyses, recent studies in our laboratories have shown that 1,25(OH)$_2$D$_3$ inhibits the growth of multiple ovarian cancer cell lines, including OVCAR3, CAOV3, A2008, etc. (6), by causing cell cycle arrests at G1-S (6) and G2-M (7) checkpoints. These findings provide a molecular foundation for further investigation of the in vivo growth-suppressing effects of 1,25(OH)$_2$D$_3$ compounds.

Because the concentration that induces the growth suppression of ovarian cancer cell lines is expected to cause hypercalcemia in vivo, 1,25(OH)$_2$D$_3$ cannot be directly used clinically to treat ovarian cancer patients. Chemical modifications of the 1,25(OH)$_2$D$_3$ have yielded several analogues that display enhanced tumor-suppressing but less calcemic activity (8). Among these is EB1089, a deltanoid derivative generated by Leo Pharmaceutical Products (Ballerup, Denmark) through structural alteration of the side chain of 1,25(OH)$_2$D$_3$ (9). EB1089 has been shown to suppress the growth of prostate (10, 11), breast (12), colon (13), and retinoblastoma (14) tumors, but the in vivo response of human ovarian cancer xenografts to EB1089 has not been examined.

In this article, we report that EB1089 inhibits the growth of OVCAR3 xenograft tumors in nude mice without causing hypercalcemia. Further analysis indicates that the suppression of the tumor growth in vivo is due to the combined effect of EB1089 in inhibiting cell proliferation as well as inducing apoptosis.

MATERIALS AND METHODS

Materials. 1,25(OH)$_2$D$_3$ was purchased from Calbiochem (La Jolla, CA). EB1089 (seocalcitol) and corresponding placebo were generously provided by Leo Pharmaceutical Products.

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Anti-human Ki-67 antibody was purchased from BD PharMingen, Inc. (San Diego, CA).

**Cell Assays for Growth, Apoptosis, and Transcriptional Activation of the GADD45 Reporter.**

OVCAR3 human ovarian cancer cells (HTB-161, obtained from American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 supplemented with 15% fetal bovine serum (Life Technologies, Grand Island, NY), 2 mmol/L L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 10 μg/mL bovine insulin. BG-1 cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum.

To determine cell numbers, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done as described (15). Absorption at 595 nm (A595 nm) was measured on a MRX microplate reader (Dynex Technologies, Chantilly, VA). Cell numbers were calculated based on a standard curve. Apoptosis was determined by flow cytometry analysis after cells were stained with Annexin V-FITC and propidium iodide following manufacturer’s instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Transcriptional activation of the vitamin D receptor was done as described (6). Briefly, 4 hours post-transfection with GADD45Luc and pCMVɣal. OVCAR3 cells were treated with vehicle, EB1089, or 1,25(OH)2D3 in fresh media for 36 hours. Cells were harvested and luciferase and β-galactosidase activities were determined as described (6).

**Nude Mouse Studies of OVCAR3 Tumor Xenografts.**

All mice were handled according to the Guide for the Care and Use of Laboratory Animals. Mice studies were carried out following the procedures approved by the Institutional Animal Care and Use Committee at University of South Florida. For inoculation into nude mice, OVCAR3 cells were washed with PBS, digested with trypsin, resuspended in RPMI 1640 containing fetal bovine serum, and pooled. After centrifugation, cells were resuspended in Matrigel (BD Biosciences Discovery Labware, Bedford, MA)-RPMI 1640 (1:1) at a concentration of 5 × 106 cells per 100 μL. Cell/Matrigel mixture of 100 μL was injected s.c. into 6-week-old female athymic nu/nu mice (Harlan Sprague-Dawley, Indianapolis, IN) on the dorsal surface. The mice were fed a vitamin D–deficient diet supplemented with 0.47% calcium (Harlan Teklad, Madison, WI) for the duration of the study. Tumor volumes were monitored by caliper measurement of the length and width and calculated using the formula of length × width × 0.5 the greater of length or width. Treatment was begun when tumors reached volumes of ~150 mm3 in average, which took ~4 weeks. Mice were randomized and treated daily by gavage with placebo or EB1089 at 0.3 or 1.0 μg/kg body weight in a volume of 20 μL. Tumor volumes and body weights were monitored every 5 days over the course of treatment. Mice were sacrificed after 30 days of treatments and tumors were removed and fixed in 10% neutral buffered formalin for histologic and immunohistochemical analyses.

**Calcium Measurements.** To determine the effect of active vitamin D compounds on serum calcium, nude mice without tumor xenografts were treated with placebo, EB1089, or 1,25(OH)2D3. Blood samples were obtained from the retro-orbital sinus at 0, 15th, and 30th days of the treatment. Blood samples from three mice were used for each group. Serum calcium was measured as a paid service by Antech Diagnostics (Southaven, MS) using a colorimetric method on Hitachi 747 analyzer. The measurement was repeated twice.

**Tumor Histology and Immunohistochemical Analysis.** Tumors were embedded in paraffin, sectioned at 5 μm, and stained with H&E. Cell proliferation was assessed by quantification with Ki-67 immunohistochemistry. The anti-human Ki-67 antibody was 1:400 diluted and immunostaining was done on the DAKO Autostainer using the Chemicon Mouse-to-Mouse Peroxidase Detection kit (Serologicals, Inc., Temecula, CA). Ki-67-positive cells were scored by visual examination of 40 randomly selected fields of ×400 magnification containing at least 2,000 cells.

**Tissue Terminal Deoxynucleotide Transferase–Mediated dUTP Nick End Labeling Assay.** Histologic analysis of DNA fragmentation was used to identify apoptotic cells in paraffin sections of the xenograft tumors. In situ terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was done using the Apoptag Peroxidase In situ Apoptosis Detection Kit (Serologicals). TUNEL-positive cells were quantified in 40 randomly selected high-power fields (×400) of each tissue section.

**RESULTS**

**EB1089 Suppresses the Growth of Ovarian Cancer Cells and Transcriptionally Activates the GADD45 Reporter Gene at Concentrations Lower Than 1,25(OH)2D3.** Before investigating the in vivo response of ovarian tumor xenografts to EB1089, we first examined the activity of EB1089 in suppressing the growth of ovarian cancer cells in vitro using 1,25(OH)2D3 as a control. OVCAR3 and BG-1 cells were treated with vehicle, 1,25(OH)2D3, or EB1089 at indicated concentrations and the cell growth was determined in MTT assays. As shown in Fig. 1A and B, 1,25(OH)2D3 at 10−7 mol/L caused a significant decrease in the growth of OVCAR3 and BG-1 cells after treatment for 6 days (43.9 ± 7.8% for OVCAR3 and 62.4 ± 17.7% for BG-1) and 9 days (59.8 ± 5.3% for OVCAR3 and 71.1 ± 14.2% for BG-1). The inhibition was not observed for 1,25(OH)2D3 at 10−8 or 10−9 mol/L, confirming our data from previous studies (7). In contrast to 1,25(OH)2D3, EB1089 at 10−8 mol/L inhibited the growth by 35.9 ± 9.6% and 43.1 ± 5.0% for OVCAR3 and by 55.4 ± 23.6% and 66.3 ± 14.7% for BG-1 after 6- and 9-day treatments, respectively (Fig. 1A), which is comparable with the inhibition by 1,25(OH)2D3 at 10−7 mol/L. The data suggest that EB1089 is 10 times more active than 1,25(OH)2D3. EB1089 at 10−7 mol/L inhibited growth by 39.1 ± 4.1% and 49.4 ± 6.9% for OVCAR3 and by 57.6 ± 29.1% and 63.4 ± 18.6% for BG-1 for 6- and 9-day treatments, respectively. The magnitude of the growth inhibition did not surpass that induced by 1,25(OH)2D3 at 10−7 mol/L or EB1089 at 10−6 mol/L, suggesting the existence of a saturation point for 1,25(OH)2D3, similar to 1,25(OH)2D3, as a chronic process, although its effective concentration is lower than the 1,25(OH)2D3.
Our previous studies have identified GADD45 as a primary target gene for 1,25(OH)2D₃, which specifically mediates the inhibitory effect of the hormone on cell cycle progression through the G2-M checkpoint (6). The induction of GADD45Luc by 1,25(OH)2D₃ is mediated through an exonic vitamin D response element that binds the vitamin D receptor (6). Thus, GADD45Luc provides a measurement for the transcriptional activation of vitamin D receptor. To test whether EB1089 regulates the transcriptional activation of the vitamin D receptor more effectively than 1,25(OH)2D₃, the activation of the GADD45Luc reporter by the natural and synthetic 1,25(OH)2D₃ compounds was compared in transient transfection assays. Consistent with the growth analyses, the reporter activation by EB1089 was more efficient than 1,25(OH)2D₃ at most concentrations, but no difference was detected for the maximal activation induced at the saturating concentration (Fig. 1C).

Besides cell cycle arrests at G1-S and G2-M checkpoints, our studies showed that treatment of OVCAR3 cells with 1,25(OH)2D₃ for 6 days induces apoptosis (Fig. 1D). To determine whether EB1089 also induces apoptosis in ovarian cancer cells, the apoptotic index of OVCAR3 cells treated with vehicle or EB1089 was determined by flow cytometry after staining with Annexin V-FITC and propidium iodide. As shown in Fig. 1D, OVCAR3 cells treated with 10⁻¹⁰ mol/L EB1089 or 1,25(OH)2D₃ and subjected to flow cytometry analysis after staining with Annexin V-FITC and propidium iodide. Analysis in duplicate; data were reproduced twice.

Fig. 1 Effects of EB1089 (EB) and 1,25(OH)2D₃ (VD) on the proliferation and apoptosis of ovarian cancer cells. OVCAR3 cells (A) and BG-1 cells (B) were plated in 96-well plates and treated as indicated. Cell growth was analyzed with MTT assays and eight samples were averaged; data were reproduced thrice. *, P < 0.01, independent-samples t test compared with ethanol (EtOH) control. C, OVCAR3 cells transfected with 0.2 μg GADD45Luc and 0.05 μg pCMVgal were treated as indicated. Luciferase activity was determined and normalized with cognate β-galactosidase activity. Analysis in duplicate; data were reproduced twice. D, OVCAR3 cells were treated with 10⁻¹⁰ mol/L EB1089 or 1,25(OH)2D₃ and subjected to flow cytometry analysis after staining with Annexin V-FITC and propidium iodide. Analysis in duplicate; data were reproduced twice.

Overall, the above analyses show that EB1089 is transcriptionally more active than 1,25(OH)2D₃, inhibits the growth of ovarian cancer cells at lower concentrations, and induces apoptosis in a similar way as 1,25(OH)2D₃. The analyses argue strongly for further investigation of its in vivo effects in whole animal experiments.

EB1089 Inhibits the Growth of OVCAR3 Tumor Xenografts in Nude Mice without Causing Hypercalcemia. After the growth-suppressing activity of EB1089 was verified in vitro using OVCAR3 and BG-1 cells, we next investigated its in vivo effect on the growth of OVCAR3 tumor xenografts as described in MATERIALS AND METHODS. As shown in Fig. 2, the size of OVCAR3 tumor xenografts treated with the placebo increased proportionally during the treatment. Treatment with EB1089 at 0.3 or 1 μg/d/kg body weight almost completely suppressed the growth of the tumors. In comparison with the placebo controls, the tumor suppression by EB1089 at both concentrations after 30 days reached statistical significance (P < 0.01). In contrast, no significant difference was observed between the size of the tumors treated with EB1089 at 0.3 and 1 μg/d/kg for 30 days (P = 0.246). Due to the heterogeneity of the tumor sizes to begin with, there is a significant variation among the tumor volumes in each group, but the suppressive effect of EB1089 on the tumor growth is obvious when the representative tumors from the placebo and treated groups were placed side-by-side to compare their size (Fig. 2B).

Mice treated with EB1089 at both doses did not show signs of morbidity during the treatment. Their body weight was not affected by the treatment with EB1089 either (data not shown). Because active vitamin D compounds are known to increase the level of blood calcium causing hypercalcemia, we next examined the effects of EB1089 and 1,25(OH)2D₃ on blood calcium. Separate groups of nude mice was treated with 1,25(OH)2D₃ and...
EB1089 and the serum calcium was measured at 0, 15th, and 30th days of the treatment. As shown in Fig. 2C, although EB1089 increased the level of serum calcium in a dose-dependent manner, it did not cause clinical hypercalcemia. In contrast, 1,25(OH)2D3 at 1.0 μg/d/kg induced hypercalcemia as expected.

**EB1089 Alters Tumor Histology and Decreases Proliferation but Increases Apoptosis of Ovarian Tumor Cells In vivo.** EB1089 has been shown to change the histology of prostate tumor xenografts (11). To investigate the effect of EB1089 on the histology of OVCAR3 xenografts, tumor sections taken from mice treated with placebo or EB1089 at 0.3 or 1.0 μg/d/kg were stained with H&E. As shown in Fig. 3, the histologic appearance of tumors from mice treated with placebo (Fig. 3A) is quite different from that of mice treated with EB1089 (Fig. 3C). Tumors from placebo controls were composed primarily of epithelial cells (Fig. 3A). Mitotic figures are frequently visible (Fig. 3C) and apoptotic cells are rare. In contrast, mice treated with EB1089 at 0.3 μg/d/kg (data not shown) and 1.0 μg/d/kg (Fig. 3B) shared a histology that showed an increased proportion of noncellular stroma components. Compared with placebo controls, mitotic figures were fewer, whereas apoptotic cells with condensed or pyknotic nuclei and eosinophilic cytoplasm were more frequently seen (Fig. 3D).

To quantitatively compare the proliferation and apoptotic index of tumors treated with placebo and EB1089, the tumor sections were stained for expression of Ki-67 and DNA fragmentation by tissue TUNEL assays, respectively. As shown in Fig. 4A, treatment with EB1089 decreases the number of Ki-67-positive cells in tumors (Fig. 4A(2)) compared with placebo controls (Fig. 4A(1)). Based on the counting of 40 randomly selected microscopic fields, the proliferation index was decreased from 82.4 ± 5.5% in the placebo controls to 45.6 ± 3.9% in

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**Fig. 2** Effect of EB1089 on the growth of OVCAR3 tumor xenografts and the level of serum calcium of nude mice. A. nude mice bearing OVCAR3 tumor xenografts were treated daily with placebo or EB1089 at 0.3 μg/d/kg (EB 0.3) or 1.0 μg/d/kg (EB 1.0). Percentage of tumor volume was calculated by dividing the volume at each time point with the average volume of the tumors at the time when the treatment begins. Points, average percentage for two independent experiments involving 21 tumors of placebo, 19 tumors of 0.3 μg/d/kg EB1089, and 21 tumors of 1.0 μg/d/kg EB1089; bars, SE. *, P < 0.01, independent-samples t test compared with placebo control. B, representative pictures of OVCAR3 tumors removed from nude mice after 30 days of treatment with either placebo or EB1089 at 0.3 or 1.0 μg/d/kg. C, blood calcium levels were determined in serum samples from nude mice treated with EB1089 or 1,25(OH)2D3 for indicated times. Data were reproduced twice. Note that the reference range for serum calcium in mice is 7.0-11.0 mg/dL.

**Fig. 3** Effect of EB1089 on the tumor histology of OVCAR3 xenografts. Representative images of tumor sections stained with H&E were captured through a computer attached to a microscope. A and C, placebo-treated tumors; B and D, tumors treated with EB1089 for 30 days. Asterisk, noncellular stromal areas; solid arrows, mitotic figures; open arrows, apoptotic cells. Magnification, ×100 for A and B and ×400 for C and D.
EB1089-treated tumors (Fig. 4A(3)). In contrast to the proliferation index, EB1089-treated tumors compared with placebo controls (Fig. 4B(1)) showed increased numbers of apoptotic cells with condensed and irregularly shaped nuclei, staining positively for TUNEL (Fig. 4B(2)). Based on the counting of randomly selected microscopic fields, the number of apoptotic cells was increased from 18 ± 5 per 10 high-power microscopic fields in placebo controls to 57 ± 6 in EB1089-treated tumors (Fig. 4B(3)). The changes in both proliferation and apoptosis indexes were statistically significant (P < 0.001 and P = 0.001, respectively).

DISCUSSION

In the present studies, we have shown for the first time that the synthetic 1,25(OH)2D3 analogue EB1089 significantly suppresses the growth of ovarian cancer in vivo in nude mice without causing hypercalcemia. We also showed that the natural and synthetic 1,25(OH)2D3 compounds induce apoptosis of human ovarian cancer cells in vitro and in nude mice. In combination with the data from our earlier studies indicating that 1,25(OH)2D3 induces cell cycle arrest (6, 7) and apoptosis (16) in ovarian cancer cell lines, the current observation that EB1089 decreases the proliferation index and increases cell death contribute to the antitumor activity of EB1089 against OVCAR3 xenografts. Our cell growth assays and reporter gene analysis showed that EB1089 is more potent than 1,25(OH)2D3 (Fig. 1), which concurs with data recently reported for OVCAR3 cells (17). The fact that the growth suppression of OVCAR3 xenografts by EB1089 was observed at 0.3 µg/d/kg, which is lower than the dose (0.5-1.0 µg/d/kg) used for other tumors (10–14), suggests that ovarian cancer might be more sensitive than other cancers to the growth suppressive effect of EB1089. Because the use of EB1089 at a higher dose such as 1.0 µg/d/kg did not further enhance the tumor-suppressing effects but caused an increase in serum calcium, it is critical to determine the lowest dose of EB1089 that is effective in suppressing the growth of ovarian cancer but with minimal effect on the level of blood calcium.

Although EB1089 was found to induce apoptosis in vitro and in vivo, it did not cause regression of OVCAR3 tumor xenografts during a 4-week treatment as judged by the measurement of tumor volumes. Histologic examination showed that EB1089-treated tumors contained more prominent acellular stroma compared with placebo controls, suggesting that the tumors may have undergone actual regression that was not reflected by simply measuring the tumor volume. Because a chemopreventive role of 1,25(OH)2D3 compounds has been suggested, the early application of EB1089 during tumor development may have allowed the detection of more dramatic in vivo antitumor effects or may have prevented the formation of tumors altogether. Furthermore, the combination of EB1089 with other therapeutic agents may clinically achieve more desirable therapeutic effects.

In summary, our study strongly argues for further investigation of EB1089 and other synthetic 1,25(OH)2D3 analogues in the therapeutic treatment of ovarian cancer patients. In addition, EB1089 has been reported to regulate genomic
stability (18, 19), to suppress angiogenesis (20), and to inhibit in vivo tumor metastasis (21). Further studies may reveal more significant applications for synthetic 1,25(OH)2D3 compounds in the clinical management of ovarian cancer.

ACKNOWLEDGMENTS

We thank Dr. L. Binderup (Leo Pharmaceutical Products) for providing the placebo, EB1089, and 1,25(OH)2D3 compounds formulated for animal studies and Paul Byvoet for the critical reading of the article.

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