Inhibition of Hepatocellular Cancer by EB1089: 
**In Vitro and In Vivo Study**

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**Abstract.** Background: Despite significant antiproliferative potential, clinical application of 1,25 dihydroxy vitamin D₃ [1,25(OH)₂VD₃] in the treatment of cancer has been hindered due to the development of hypercalcemia. Various derivatives of 1,25(OH)₂VD₃ have been synthesized to overcome this side-effect. Seocalcitol (EB1089) is a vitamin D analog that has been extensively studied and shown to have profoundly reduced hypercalcemic effects. Here the effects of EB1089 were evaluated in Hep 3B, SKHEP-1, PLC/PRF/5, HTC and Novikoff hepatocellular cancer (HCC) cell lines. Materials and Methods: In vitro, cells were treated with different concentrations of EB1089 (1-1000 nM). Analytical tests were then performed including cell count and ³H thymidine assay. For in vivo analysis, SKHEP-1 cells were xenografted into nude male mice. Twenty-four hours after inoculation, mice were randomly assigned to a control group (n=10) or one of the treatment groups (3 groups of 10 mice) receiving 0.02, 0.1 or 0.5 μg/kg/day of EB1089. Control animals received the vehicle (propylene glycol). To minimize the number of intraperitoneal injections, oral and intraperitoneal routes were used on alternate days. Tumor size was measured every third day and the volumes were estimated using the formula 0.5 × length × (width)². Results: In vitro: Proliferation of Hep 3B, PLC/PRF/5 and SKHEP-1 HCC cells was significantly inhibited at all EB1089 concentrations tested, while HTC cells only responded to 1,000 nM concentration of EB1089. Proliferation of Novikoff cells was unaffected by the drug at all concentrations examined. In vivo: EB1089 effectively inhibited SKHEP-1 tumor growth without inducing hypercalcemia (p<0.05). Conclusion: Results of the present study indicate that EB1089 is an effective growth inhibitor of HCC tumors.

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world and the third most common cause of cancer-related death (1). Over 600,000 new cases of liver cancer are diagnosed globally each year, with a five-year survival rate of 11%. It accounted for 7.6 million deaths in 2005, accounting for approximately 13% of deaths overall (1, 2). The outcome of patients presenting with HCC varies according to the stage of the disease and the treatment applied. For patients with preserved liver function and no portal hypertension who develop HCC that is confined to one region of the liver, resection is the preferred treatment. If resection is not possible because of poor liver function and the HCC is within the Milan criteria (1 nodule ≥5 cm, 2-3 nodules ≥3 cm), liver transplantation is the treatment of choice (3). Despite therapeutic advances, there has not been significant improvement in the overall survival of patients who have HCC in the last two decades (4). At the time of diagnosis, more than 80% of patients present with multicentric HCC and advanced liver disease or comorbidities that restrict the therapeutic measures to best supportive care. Therefore, early diagnosis, the development of novel systemic therapies for advanced disease and HCC prevention are of paramount importance (5).

Considerable evidence suggests that 1,25 dihydroxy vitamin D₃ [1,25(OH)₂VD₃] may play an important role in the treatment of cancer. Inhibition of proliferation by 1,25(OH)₂VD₃ has been demonstrated for breast (6, 7), prostate (8, 9), colon (10, 11), ovarian (12, 13), melanoma and pancreatic carcinoma (14). Furthermore, xenografts of colon, melanoma, prostate and pancreatic carcinoma cell lines in immunodeficient mice have been shown to regress following treatment with 1,25(OH)₂VD₃ (15). Although the therapeutic potential of 1,25(OH)₂VD₃ is encouraging, its use as a systemic anticancer agent has been limited due to the induction of hypercalcemia (16). To overcome this...
problem, large numbers of synthetic analogues of vitamin D have been developed. Seocalcitol (EB1089), a synthetic vitamin D analog, exhibits reduced hypercalcemic activity relative to 1,25(OH)₂D₃ in vivo. Many studies have shown the antiproliferative activity of EB1089 in several in vitro and in vivo experiments (17-22). Vitamin D receptor (VDR)-mediated antiproliferation has been reported in the Hep G2 cell line while a HCC cell line did not respond due to absence of VDR (23). In another study, long-term intraperitoneal (IP) administration of EB1089 at a dose of 0.5 μg/kg body weight every other day in C3H/Sl mice exerted a very strong inhibitory effect on HCC development (24). In this study, we targeted five different HCC cell lines (Hep 3B, SKHEP-1, PLC/PRF/5, HTC and Novikoff) to determine the in vitro effects, while SKHEP-1 was selected for in vivo study.

Materials and Methods

HCC cell lines (Hep 3B, SKHEP-1, PLC/PRF/5, HTC and Novikoff) were obtained from the European Collection of Cell Cultures (UK). Cell lines were then grown in Eagle’s minimum essential medium (MEM) and Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 units/ml each of streptomycin and penicillin. EB1089 was prepared in ethanol and then diluted in medium to give the final desired concentration of drug and an ethanol concentration of 0.1%.

³H Thymidine assay. For ³H thymidine assay, different concentrations of EB 1089 were prepared in medium. A total of 10,000 cells per well was placed in 24-well plates and was treated with different concentrations of EB1089. Media were replaced every second day. At the end of the treatment period (5 days), cell cultures were assayed for thymidine incorporation by the addition of 0.5 μCi of ³H thymidine (60 Ci/mmol. ICN Biochem, Irvine, CA) to each well for the last 4 h of culture. The amount of radioactivity incorporated into cells was determined using a β-scintillation counter. Results are presented as percentage ³H thymidine incorporation relative to control.

Cell count. A total of 5,000 cells per well was plated in 6-well plates. The cell treatment procedure was similar to the ³H-thymidine assay as described above. At the end of the treatment period (8 days), cells were trypsinized and counted with a hemocytometer. In all experiments, cells treated with the medium containing 0.1% ethanol were taken as control for EB1089-treated cells. All counts were obtained in quadruplicate and each experiment was repeated at least twice.

Xenografts in nude mice. Six- to eight-week-old male BALB/c Nu/Nu mice (Animal Resources Center, Perth, Australia) were inoculated subcutaneously with 10⁶ SKHEP-1 cells into the right flank. Twenty-four hours after inoculation, mice were randomly assigned to a control group (n=10) or the treatment groups (n=10), receiving 0.02, 0.1 or 0.5 μg/kg per day of EB1089 (intraperitoneal or oral on alternate days). Control animals received propylene glycol alone. Tumor size was measured using vernier calipers every third day and the volumes were estimated using the formula 0.5 x length x (width)² (14). Animals received sterile food and water ad libitum.

For determining serum calcium levels, 0.5 ml of blood was taken under general anesthesia at the end of the experiment using cardiac puncture. Calcium levels were determined by the o-cresolphthalein complex one method according to manufacturer’s instructions (Boehringer Mannheim Automated Analysis, Hitachi system 717; Boehringer Mannheim, Australia). At the conclusion of the experiment, mice were sacrificed with a lethal intraperitoneal dose of sodium pentobarbitone and the tumors were excised.

Statistical analysis. For the in vitro work, the differences between groups were analyzed by one way analysis of variance. Tumor volume data were analyzed using two-way analysis of variance followed by Tukey’s multiple comparison tests. Data are presented as mean ± standard error of mean (SEM). A p-value of <0.05 was considered to be significant.

Results

³H Thymidine assay. Profound dose-dependent inhibition of cellular growth was seen with Hep 3B cells when treated with EB1089. Results obtained in the ³H thymidine incorporation assay with hepatoma cell lines are presented in Figure 1. Treatment with a concentration of 1,000 nM of EB1089 resulted in significantly reduced ³H thymidine incorporation in these cells to 2.7±0.4% that of the control (p<0.001). Greater than 50% inhibition was present even at the lower concentration of 1 nM (p<0.001). SKHEP-1 was also inhibited by EB1089; however, significant inhibition by ³H thymidine incorporation was observed only at the higher concentrations (100 and 1000 nM). In SKHEP-1 cells, the IC₅₀ for EB1089 was 460.99 nM. A modest inhibition in cellular growth was observed with PLC/PRF/5 cells when treated with EB1089. Maximum inhibition was observed at the highest concentration (1,000 nM) of EB1089 (70±10.5%, p<0.001). Compared to human cell lines, the rat hepatoma cell lines, Novikoff and HTC, displayed much lower sensitivity to EB1089 treatment. Our previous results have shown the absence of VDR in Novikoff cells (25).

Cell count. Results obtained in the cell count study for the human cell lines Hep 3B, SKHEP-1 and PLC/PRF/5 showed a similar pattern to that seen in the ³H thymidine assay Figure 2. Cell proliferation was most effectively inhibited in Hep3B cells, resulting in dose-dependent (p<0.001) reduction in the number of cells remaining after the EB1089 treatment. In SKHEP-1 cells, a significant reduction in the cell number was observed at 100 nM concentration of EB1089.

In vivo study. For our in vivo study SKHEP-1 was chosen because it has been reported to be the most tumorigenic human cancer cell line in nude mice (26). The response to EB1089 treatment obtained in nude mice bearing SKHEP-1 tumor was similar to the data obtained in the cell culture work. Profound inhibition of tumor growth was observed at 0.02, 0.1
and 0.5 μg/kg/day dose Figure 3. However, there was no significant difference in growth inhibition between the three groups treated with EB1089. No significant increase in calcium level was observed during the study period with 0.02, 0.1 and 0.5 μg/kg/day dose of EB1089.

**Discussion**

Although the active form of vitamin D, 1,25(OH)2VD3, is a potent cell-differentiating agent, its use in cancer prevention or therapy is precluded because of its hypercalcemic effects (16). It has been shown in experiments that EB1089 has a tissue-specific uptake which differs from that of 1,25(OH)2VD3. Although the half lives of EB1089 and 1,25(OH)2VD3 are comparable, their initial/apparent volume of distribution is different, which is demonstrated by their different concentrations after intravenous administration of the same dose to rats (27). EB1089 has also been shown to be approximately 50 to 80 times more potent than 1,25(OH)2VD3 in inhibiting cancer cell growth in vitro and it has markedly reduced action on calcium metabolism in vivo (28, 29). It has been shown that the inhibition of cell growth in response to 1,25(OH)2VD3 involves cell cycle arrest in the G1-phase. Treatment of Hep G2 cells with 1,25(OH)2VD3 also resulted in cell cycle arrest in G0/G1 (30). EB1089 has been shown to induce apoptosis and elevation of expression of p27Kip1 and PTEN protein (31). Potential mechanisms reported for the selectivity of EB1089 include altered systemic transport via vitamin D-binding protein (DBP), a change in cellular uptake of EB1089, metabolism to active and non-active compounds, induction of an altered conformational change in the VDR that could influence heterodimerization with retinoid X receptor (RXR), binding to DNA and subsequent recruitment of other components to the transcriptional initiation complex (32, 33).
We observed a potent inhibitory action of 1,25(OH)₂VD₃ and its analogues EB1089 and CB1093 on the cellular growth of Hep G2 cells (25): Hep-3B cells showed extensive sensitivity to EB1089, similar to the response seen with Hep G2 cells. In Hep 3B cells, a significant inhibitory effect of EB1089 was seen at concentrations as low as 0.1 nM. PLC/PRF/5 and SKHEP-1 cells were not affected by the lower concentrations of the drug, while they were modestly inhibited at the higher concentrations. HTC and Novikoff cells were least affected. Another interesting observation is the higher inhibition by EB1089 with a lower tumor volume, indicating that EB1089 may be more efficacious in the initial stages of tumor development or while the tumor size is small. In vitro experiments showed the highest inhibition with Hep 3B cells but the initial amount of incorporation of 3H thymidine is also lowest in these cells. In vivo experiments also showed the highest level of tumor inhibition with the smallest initial tumor size at a concentration of 0.02 μg/kg/day. Both of these observations support the idea that EB1089 is more effective with small tumor size.

EB1089 was more potent than 1,25(OH)₂VD₃ as reported in a previous study (28). Results obtained in the cell count study for the Hep 3B and SKHEP-1 cells confirmed the inhibitory effect seen in the ³H-thymidine proliferation study. 1,25(OH)₂VD₃ produces biological responses via the genomic pathway and VDR has been detected on many cell types. We have previously reported that liver cancer cell lines do possess VDR and the VDR expression was highest in Hep G2 cells, the most sensitive to the antiproliferative effects of 1,25(OH)₂VD₃ and EB1089. Interestingly, no VDRs were detected in Novikoff cells, which failed to respond to 1,25(OH)₂VD₃ and, in this study, to EB1089 (30). Miyaguchi and Watanabe also reported dose-dependent inhibition of the human hepatoma cell line PLC/PRF/5 by 1,25(OH)₂VD₃. Inhibition of cell proliferation was reported to be tightly linked to mechanisms that regulated cell cycle progression (34).

In a phase II study of EB1089 in HCC patients, less than a 20% objective response was reported, with minimal side-effects. The dose of EB1089 was titrated in this study based on calcium levels; most of the patients tolerated a daily dose of 10 μg (35). In summary, EB1089 has been found to be more efficient than 1,25(OH)₂VD₃ in inducing apoptosis in various cancer cells in vitro and in vivo. Its specificity for cancer cells and lower hypercalcemic effect further augment the possibility of its clinical use. Our study supports the role of EB1089 in the treatment of HCC. However, further studies are warranted to elucidate its efficacy and toxicity.

References


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