A Calcitriol Analogue, EB1089, Inhibits the Growth of LNCaP Tumors in Nude Mice

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Abstract

Limited options for the treatment of prostate cancer have spurred the search for new therapies. One innovative approach is the use of 1a,25-dihydroxyvitamin D3 (calcitriol) analogues to inhibit cancer growth. We demonstrate here that the calcitriol analogue, EB1089, extensively inhibits the growth of LNCaP prostate cancer cells in culture and causes the cells to both accumulate in G2-G3 and undergo apoptosis. Importantly, we found that EB1089 inhibits the growth of LNCaP tumor xenografts in nude mice. Because of these antiproliferative properties in vivo, EB1089 is a potential new therapeutic agent for the treatment of prostate cancer.

Introduction

The lack of effective treatments for advanced prostate cancer has spurred research toward the development of novel chemotherapeutic and chemopreventive methods to treat the disease. Calcitriol1 regulates bone maintenance and calcium metabolism, but more recently, calcitriol has been shown to have significant antiproliferative properties when administered to many cancer cell lines in vitro, including the LNCaP prostate cancer cell line (1). Although calcitriol strongly inhibits LNCaP cell growth in culture, the concentrations required (~10–100 nM; Ref. 1) are unachievable in vivo because of unacceptable side effects such as hypercalcemia (2). Much effort has been devoted to the development of analogues of calcitriol that retain antiproliferative capabilities but do not cause unwanted side effects in vivo. One promising analogue, EB1089 (3), developed by Leo Pharmaceuticals (Ballerp, Denmark), is a deltanoid derivative of calcitriol that inhibits LNCaP cell growth at much lower concentrations than calcitriol (~1 nM; Ref. 4). We demonstrate here that EB1089, at much lower levels than those required for calcitriol, virtually irreversibly inhibits the growth of LNCaP cells and causes a comparable amount of apoptosis in G2-G3 cell cycle accumulation. We also show that pharmacological levels of EB1089 injected i.p. substantially reduce the growth of LNCaP tumors in a nude mouse xenograft model without raising serum calcium levels (a measure of hypercalcemia) beyond the normal range. This property of EB1089 makes it a promising candidate for the treatment of prostate cancer.

Materials and Methods

Compounds. Calcitriol was obtained from Solvay DuPhar (Weesp, the Netherlands), and EB1089 (3) was generously provided by Dr. Lise Binderup of Leo Pharmaceuticals. Both compounds were dissolved in ethanol and stored in the dark at ~80°C. All other chemicals were reagent grade unless otherwise stated.

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3 The abbreviations used are: calcitriol, 1α,25-dihydroxyvitamin D3; AR, androgen receptor.
EB1089 inhibits LNCaP prostate tumor growth

**Results and Discussion**

**Growth-inhibitory Properties of EB1089.** We have shown previously that 1 nM EB1089 is as effective as 100 nM calcitriol at inhibiting the growth of LNCaP cells in culture (5). To determine whether the growth-inhibitory effects extend beyond the period of treatment, we compared, over a 12-day period, the growth rates of cells treated with EB1089 for only 6 days to growth rates of cells continuously treated with EB1089 and cells treated with vehicle alone. The ability of a 6-day treatment with EB1089 to completely halt growth of the cells and to prevent the capacity to proliferate during a 6-day recovery period (Fig. 1) illustrates the remarkable antiproliferative capabilities of 1 nM EB1089.

**EB1089 Causes Both Cell Cycle Arrest and Apoptosis.** Treatment of LNCaP cells with high concentrations of calcitriol causes G0-G1 accumulation (5, 6) and induces apoptosis (7, 8). To determine whether 1 nM EB1089 was capable of inducing comparable responses, we first examined the cell cycle distribution of EB1089-treated LNCaP cells. We found that treatment with either 1 or 10 nM EB1089

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% G0-G1</th>
<th>% Td labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>22.4 ± 16.8</td>
<td>3.0 ± 1.6</td>
</tr>
<tr>
<td>1 nM calcitriol</td>
<td>26.5 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>10 nM calcitriol</td>
<td></td>
<td>17.1 ± 4.3a</td>
</tr>
<tr>
<td>100 nM calcitriol</td>
<td>23.2 ± 0.9a</td>
<td></td>
</tr>
<tr>
<td>0.1 nM EB1089</td>
<td>42.1 ± 1.1a</td>
<td></td>
</tr>
<tr>
<td>1 nM EB1089</td>
<td>85.3 ± 1.6b</td>
<td>22.9 ± 6.5a</td>
</tr>
<tr>
<td>10 nM EB1089</td>
<td>89.0 ± 1.5a</td>
<td></td>
</tr>
</tbody>
</table>

a P ≤ 0.05 compared with ethanol sample.
b P ≤ 0.05 compared with same concentration of calcitriol.

Laboratories, Inc., Burlingame, CA). Human androgen receptor antibody AR441 was used to detect AR-positive cells in tumor sections. Positive staining was visualized using the Vectastain kit reagents and 3,3'-diaminobenzidine (Research Genetics, Huntsville, AL). Slides were counterstained with hematoxylin and dehydrated. Images were recorded using a Zeiss Axioskop light microscope at ×10.
causes a substantial accumulation of the cells in G_0-G_1 (shown in Table 1), whereas our previous studies demonstrated that 100 nM calcitriol was required to achieve similar levels of accumulation (5).

To determine whether EB1089 also caused LNCaP cell apoptosis, we assessed the amount of DNA fragmentation characteristic of apoptotic cells after treatment with 1 nM EB1089 and found that 1 nM EB1089 is as effective as 100 nM calcitriol at causing apoptosis of LNCaP cells (Table 1). Collectively, the cell cycle and apoptosis assays show that 1 nM EB1089 induces the same LNCaP cell response as does 10 or 100 nM calcitriol.

EB1089 Inhibits the Growth of LNCaP Tumors in Nude Mice. Because LNCaP cells require additional factors or stromal cells to induce tumor growth in nude mice (9), we mixed LNCaP cells with Matrigel (10) and injected the cells s.c. into mice to produce tumors. Nude mice with established LNCaP tumors (1/mouse) were then treated with either vehicle (sesame oil) or EB1089 diluted in sesame oil for a 19-day period. Tumors in both groups started with an approximate calculated volume of 150 mm^3 (Fig. 2A), and the average final tumor volumes were 259 ± 33.7 in animals that received no EB1089 and 165.2 ± 23.1 in animals treated with EB1089, an increase in mean tumor volume of 109 and 15 mm^3, respectively. Although

Fig. 3. Analysis of LNCaP tumors. A and B, size comparison in animals receiving injections at two tumor sites and treated as described in Fig. 2. A, sesame oil; B, EB1089. Tumors from Fig. 2A were harvested, fixed, embedded in paraffin, sectioned onto poly-lysine-coated slides, and used for experiments shown in C–H. Tumor sections C, E, and G are from sesame oil-treated animals; tumor sections D, F, and H are from animals receiving EB1089. Sections C and D were stained with H&E. Immunohistochemistry was performed on sections E, F, G, and H using the human AR antibody AR441. G and H are sections incubated without AR441.
this difference is significant ($P \leq 0.05$), this is likely an underestimate of the difference in tumor burden for two reasons: (a) we are estimating the third dimension. Shown in Fig. 3, A and B are typical tumors from control and treated mice. For this particular figure, animals received injections at two sites instead of one site. Note that the control tumors appear nearly round, whereas the EB1089-treated tumors are quite flat. Hence, the actual volumes differ more than is estimated based on the volume formula (length $\times$ width $\times \frac{1}{2}$ the greater of length or width); and (b) as described below, the proportion of measured tumor volume actually occupied by tumor cells is less in the EB1089-treated tumors than in the control tumors.

**Treatment with EB1089 Alters Tumor Histology.** To compare the cellular content of each tumor, we harvested tumors from both treatment groups and stained tumor sections with H&E. As assessed by light microscopy (Fig. 3, C and D), the tumors from untreated animals (Fig. 3C) consisted of tightly packed cells, and many blood sinuses were apparent within the tumor. In contrast, tumors from treated animals (Fig. 3D) were considerably less cellular and were composed primarily of acellular material; moreover, the extent of vascularization appeared to be less than in untreated tumors. Cells were most commonly found in small islands. This histology indicated that there was a very large difference in actual tumor cell burden between treated and untreated animals.

To determine which cells were LNCaP cells, we used an antibody specific for the human AR. Fig. 3, E and F, illustrate the AR-positive LNCaP cells within both untreated and treated tumor sections. The control section (Fig. 3E) consists of densely packed AR-positive cells interspersed with some AR-negative cells. The sections from the treated animal (F) contained islands of AR-positive cells amid acellular material and regions containing AR-negative (presumably mouse) cells. Controls (incubated without the AR antibody) for specificity are shown in Fig. 3, G and H.

**Serum Calcium Levels Are in the Normal Range.** The tumor growth inhibition reported above occurred with no physically apparent symptoms of hypercalcemia (lethargy or dehydration), and serum calcium levels in both groups (9.25 $\pm$ 0.35 for untreated animals and 10.5 $\pm$ 0.60 in treated animals) were within the normal range of serum calcium for mice of the age and sex used (Fig. 2D; Ref. 11). A small but insignificant drop ($P = 0.09$) in weight was observed in animals treated with EB1089, but this did not appear to significantly affect the health of the animals (Fig. 2C). In other unrelated studies, the same dose of EB1089 administered for over 6 weeks did not raise serum calcium levels above the normal range, and normal mouse weights were maintained.

**Conclusions.** Although calcitriol itself and other calcitriol analogues have been tested in the Dunning tumor model of prostate cancer (12) and in a PC-3 human prostate cancer cell athymic mouse model (13) with some success in inhibiting prostate tumor growth, hypercalcemia and weight loss were also noted. In contrast, our findings show that EB1089 can inhibit LNCaP tumor growth without inducing hypercalcemia. Studies that report the effect of EB1089 in breast (14), pancreatic (15), and colon cancer (16) models suggest that this compound may be useful in treating a number of tumors. Two promising studies in humans demonstrated that calcitriol can stabilize rising PSA levels in prostate cancer patients with advanced disease, although both studies were limited by hypercalcemic side effects (17, 18). Of interest will be whether EB1089 has similar effects in humans without the incidence of side effects.

Our finding that the treated tumors consist of small islands of cells surrounded by acellular material and mouse-derived cells rather than large masses of tumor cells suggests that one potential mechanism of EB1089 action may be through the inhibition of new blood vessel formation. This inhibition of blood vessel formation could result in cell islands that cannot obtain enough nutrients to proliferate and eventually become necrotic. In fact, in some of our tumor sections from treated animals, we have observed necrotic centers in the cellular islands. Some studies have already reported calcitriol as having significant antiangiogenic properties (19, 20), and additional studies will be necessary to elucidate the antiangiogenic properties of EB1089.

Our findings of the antiproliferative effects of EB1089 on LNCaP xenografts with no observable calcemic side effects may offer a novel therapeutic option for the treatment of prostate cancer.

**Acknowledgments**

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**References**

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