Reversal of Hypercalcemia with the Vitamin D Analogue EB1089 in a Human Model of Squamous Cancer

Khadija El Abdaimi, Vasililiou Papavasiliou, Shafaat A. Rabbani, Johng S. Rhim, David Goltzman, and Richard Kremer

Departments of Medicine, McGill University and Royal Victoria Hospital, Montreal, Quebec H3A 1A1, Canada [K. E. A., S. A. R., D. G., R. K.], and Laboratory of Molecular Oncology, National Cancer Institute, Frederick, Maryland 21702 [J. S. R.]

Abstract

EB1089, an analogue of 1,25 dihydroxyvitamin D with low calcemic activity is a potent inhibitor of parathyroid hormone-related peptide (PTHRP) production in vitro. The purpose of the present study was to determine whether EB1089 could reverse established hypercalcemia in BALB C nude mice implanted s.c. with a human epithelial cancer previously shown to produce high levels of PTHRP in vitro. Total plasma calcium was monitored before and after tumor development and increased steadily when the tumor reached ≥0.5 cm³. When total calcium was ≥2.85 mmol/liter, animals were treated with a constant infusion of EB1089 or vehicle alone for a period of 2 weeks. A significant and sustained reduction of plasma calcium from 3.2 ± 0.1 to 2.7 ± 0.08 (P < 0.01) mmol/liter was observed during infusion with EB1089. In contrast, calcium levels in vehicle-treated animals continued to rise during the infusion period. Tumor growth velocity also slowed significantly after the administration of EB1089 as compared with vehicle-treated animals. Plasma PTHRP levels measured at the end of the 2 weeks’ infusion period were significantly lower in animals treated with EB1089 as compared with animals treated with vehicle alone (44 ± 8 pg/ml versus 194 ± 35 pg/ml, P < 0.001). These results, therefore, demonstrate that EB1089 can reverse established hypercalcemia in a human model of squamous cancer.

Introduction

Previous studies (1, 2) have clearly demonstrated that 1,25(OH)₂D₃ is a potent antiproliferative and prodifferentiative agent. These properties have been demonstrated in vitro not only in normal cells but also in cancer cells (3–5). In vivo studies (6) have also produced significant tumor regression in human tumors in nude mice. However, the therapeutic application of 1,25(OH)₂D₃ is seriously limited by its side effects, including tumor regression in human tumors in nude mice. However, the therapeutic application of 1,25(OH)₂D₃ is seriously limited by its side effects, which include hypercalcemia and hypercalcuria (7). Although its biological properties and its effect on tumor growth makes 1,25(OH)₂D₃ a potential anticancer agent, its calcium-regulating properties would normally exclude it as a candidate for treating MAH, a condition frequently encountered in hospitalized patients (8). However, paradoxically we previously hypothesized that this obstacle may indeed be overcome by using vitamin D analogues with low calcemic activity. The rationale for using these analogues to treat MAH derives from our previous in vitro and in vivo studies as outlined below.

Hypercalcemia is associated with squamous cell cancers, which typically overproduce PTHRP (9). In normal cells, we previously demonstrated that 1,25(OH)₂D₃ blocks PTHRP production (10). Using a multistep model of epithelial cell carcinogenesis, we demonstrated that the progression from the normal to the malignant phenotype was characterized by a partial resistance to the inhibitory effect by 1,25(OH)₂D₃ requiring 10- to 100-fold higher concentrations of 1,25(OH)₂D₃ to achieve the same effects (11, 12). To develop alternative strategies to block PTHRP production in vitro and in vivo, several 1,25(OH)₂D₃ analogues—known to have low calcemic activities yet to retain strong anti proliferative effects on keratinocytes in vitro (13)—were tested. One such analogue, EB1089 (Leo Pharmaceuticals Ltd, Ballerup, Denmark), has a half-life similar to that of 1,25(OH)₂D₃ yet is 10 times less potent in promoting hypercalcemia in rats (14). In the tumor progression model, EB1089 was 100 times more potent than 1,25(OH)₂D₃ in inhibiting PTHRP (14). EB1089 is, therefore, not only a potential inhibitor of PTHRP overproduction in vivo but represents a possible new strategy in hypercalcemia therapy.

Subsequently, an animal model of MAH, the rat Leydig cell tumor H500 (15) was used. The hypercalcemic state associated with this rat testicular cancer has been linked to PTHRP (16, 17). Animals that were implanted with the rat Leydig cell tumor H500 and were treated simultaneously with a constant infusion of EB 1089 maintained normocalcemia and had lower circulating PTHRP concentrations than animals treated with the vehicle alone (18). These results clearly indicated that vitamin D analogues with low calcemic activities can prevent the development of hypercalcemia in an established animal model when administered at the time of tumor implantation. However, for these analogues to be useful clinically, it remains to be determined that they can reverse established hypercalcemia and that they can be applied to human models of MAH. Our present study was designed to closely mimic the clinical situation encountered in patients with MAH. In this experimental design, a human model of squamous cancer-producing PTHRP was used, and animals were treated after the onset of hypercalcemia.

Our present data clearly indicate that EB1089 efficiently blocks PTHRP production and reverses established hypercalcemia in nude mice implanted with human squamous tumors that express high levels of PTHRP.

Materials and Methods

Cell Culture Conditions. The HPK1A cell line was established from normal human keratinocytes by stable transfection with human papillomavirus type 16 (19). Despite acquiring an indefinite life span in culture, these cells retain differentiation properties characteristic of normal keratinocytes (20) and are nontumorigenic when injected into nude mice. These immortalized cells were subsequently transformed into the malignant HPK1A-ras cell line after transfection with a plasmid carrying an activated H-ras oncogene (12, 21). In addition to forming colonies in soft agar, the malignant HPK1A-ras cells produce squamous cell carcinoma when transplanted into nude mice. HPK1A-ras cell line was seeded and grown in DMEM (Life Technologies, Inc.)

Received 2/26/99; accepted 5/28/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Medical Research Council of Canada Grant MT 10839 (to R. K.).
2 To whom requests for reprints should be addressed, at Calcium Research Laboratory, H4.67, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec H3A 1A1, Canada. Phone: (514) 843-1632; Fax: (514) 843-1712.
3 The abbreviations used are: 1,25(OH)₂D₃, 1,25 dihydroxyvitamin D₃; PTHRP, parathyroid hormone-related peptide; MAH, malignancy-associated hypercalcemia; FBS, fetal bovine serum; iPTHPR, immunoreactive PTHPR.
supplemented with 10% FBS (Life Technologies, Inc.) and maintained by serial passaging. Prior to s.c. implantation into nude mice, proliferating cells were trypsinized, washed in DMEM containing 10% FBS, and resuspended in complete medium.

For cell-growth experiments, cells were seeded at a density of $1 \times 10^4$ cells/well in 24-well cluster plates and grown to 20% confluence. After 24 h in basal conditions (DMEM without serum), fresh medium containing 10% FBS without or with varying concentrations of EB1089 was added to the cultured cells, and incubations were continued for 72 h. For survival assays, cells were treated with increasing concentrations of EB1089 without FBS. Cells were trypsinized and counted in a coulter counter (LKB, Montreal, Quebec, Canada). XTT-Microculture tetrazolium assay for cell growth was measured using a Bio-Rad microplate reader. Results were then expressed as percent of FBS-stimulated growth.

**Vitamin D Analogue.** EB1089 was kindly provided by Leo Pharmaceuticals (14). EB1089 has terminal ethyl groups and double binds (at positions 22 and 24) in the side chain. This compound has low calcemic activity (14) and a half-life similar to 1,25(OH)2D3 in vivo (Ref. 22; Table 1).

**Animal Protocols.** Balbc nude mice (20 g; female) were implanted s.c. with 107 ras-transformed keratinocytes (HPK1Aras) as described previously (21) in 200–300 μl of suspension of complete medium (DMEM and 10% FBS).

All of the animals were examined twice a week for the development of a palpable tumor at the site of injection or other s.c. sites. Three-dimensional tumor measurements were done using calipers. Tumor diameters long axis (L) and mean mid axis width (W) were measured to estimate the tumor volume using the following formula:

$$V = \frac{4}{3} \pi \left( \frac{L W}{2} \right)$$

Growth curves were generated by plotting the mean tumor volume of mice treated with EB1089 against mice treated with vehicle alone.

Preliminary experiments determined the minimum effective dosage of EB1089 that does not result in hypercalcemia in non-tumor-bearing animals. When the tumor-bearing animals developed hypercalcemia (total calcium $>2.85$ mmol/liter), osmotic minipumps (model 2004, Alza Corporation, Palo Alto, CA) were implanted under general anesthesia s.c. on the back of the animals immediately adjacent to the tumor site. Each minipump contained EB1089 dissolved in 0.1 mg/ml in polyethylene glycol: 0.05 M Na2HPO4 (80:20) to deliver a continuous dose of the compound for up to 2 weeks at a delivery rate of 2.5 μl/h. One group of tumor-bearing animals received vehicle alone. Each group consisted of eight animals.

**Plasma Calcium and PTHRP Measurements.** Plasma samples were obtained by orbital bleeding at regular intervals (every 5–7 days), and 50–100 μl were used to measure total calcium and albumin by microchemistry (Kodak Ektachrome).

Animals were killed by cardiac puncture, and 300 μl of plasma were recovered for measurement of plasma calcium and PTHRP. PTHRP was measured using an immunoradiometric assay as described previously (23). The assay recognizes the intact first 86 amino acids of the molecule and has a detection limit of 2 pg/ml.

**Statistical Analysis.** All of the results are expressed as the mean ± SE, and statistical comparisons are made on the basis of Student’s t test or a one-way ANOVA, using a Bonferroni adjustment when appropriate (24).

**Table 1** Properties of 1,25(OH)2D3 and EB1089

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative calcemic activity</th>
<th>In vivo half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)2D3</td>
<td></td>
<td>2.4 h*</td>
</tr>
<tr>
<td>EB1089</td>
<td>0.4*</td>
<td>2.8 h*</td>
</tr>
</tbody>
</table>

*Binderup et al. (14).

**Fig. 1.** Effect of EB1089 on plasma calcium in normal Balb C nude mice. Normal non-tumor-bearing animals were infused with 20, 25, or 35 pmol of EB1089 per 24 h or with vehicle alone (four animals per group) by constant infusion using Alzet osmotic minipumps. Blood was collected at timed intervals by orbital bleeding, and total plasma calcium was measured by microchemistry (Kodak Ektachem, Montreal, Quebec, Canada). Results represent the mean ± SE of three different experiments.

**Fig. 2.** Simultaneous analysis of tumor growth and plasma calcium. Balb C nude mice were implanted s.c. with 107 HPK1Aras cells as described in “Materials and Methods.” Tumor volume and plasma calcium were measured at timed intervals. Note that plasma calcium remains within the normal range until around 50 days after tumor implantation and starts to rise when the tumor size is above 500 mm3.
EB1089 IN MALIGNANT HYPERCALCEMIA

Effect on PTHRP Production. iPTHRP was measured in the plasma of non-tumor-bearing animals (control), untreated tumor-bearing animals, and hypercalcemic tumor-bearing animals treated with EB1089 or vehicle alone. iPTHRP was low and not significantly different between a group of eight non-tumor-bearing animals (controls) and a group of eight normocalcemic tumor-bearing animals (15 ± 4 pg/ml versus 18 ± 5 pg/ml; Fig. 4). Normocalcemic tumor-bearing animals with a tumor volume of < 0.5 cm³ were sacrificed 4–6 weeks after tumor implantation. All of the hypercalcemic tumor-bearing animals receiving vehicle alone and bled by cardiac puncture at the time of death had high iPTHRP plasma concentration (194 ± 35 pg/ml), whereas tumor-bearing animals receiving EB1089 and killed 2 weeks after the administration of the analogue had a significant reduction of PTHRP (44 ± 8 pg/ml, P < 0.01; Fig. 4).

Effect on Cell Proliferation in Vitro and Tumor Growth in Vivo.

To further understand the mechanism of this effect, we performed in vitro studies for cell proliferation and survival assays (to assess apoptosis). These results are summarized in Table 2. In serum-treated cells, EB1089 significantly inhibited cellular growth in a dose-dependent fashion achieving a maximal inhibition at 10⁻⁷ M. However, no effect on cell survival was detected in serum-deprived experiments (data not shown).

Tumor growth velocity was assessed before and during infusion of EB1089 or vehicle alone (Table 2). Tumors continued to grow in both treated and control group. However, in EB1089-treated animals, tumor growth velocity decreased significantly as compared with control animals (16.8% ± 5% versus 95% ± 32%; P < 0.05).

Discussion

1,25(OH)₂D₃ analogues with low calcemic activities are of potential value as anticancer agents (13, 18, 25–28). These analogues retain strong antiproliferative effects, although less calcemic than 1,25(OH)₂D₃. In previous studies, we have used one such analogue, EB1089, and demonstrated its strong capacity to inhibit PTHRP production in vitro (13) and in vivo (18). Our present data indicate that this analogue can also reverse established hypercalcemia in nude mice that have been implanted with a human squamous cancer. This tumor produces high levels of PTHRP (11, 13), a mediator linked to MAH of the majority of solid tumors in humans (9). Our previous demonstration (18) that EB1089 could be used as a preventative agent in the treatment of hypercalcemia in the rat Leydig cell tumor suggested that such an analogue could also be effective in reversing established hypercalcemia, a clinical situation frequently encountered in advanced cancer. Furthermore, a human model of epithelial carcinogenesis was chosen to closely mimic the human hypercalcemia; however, higher doses produced a progressive increase in plasma calcium (Fig. 1). Consequently, hypercalcemic tumor-bearing animals were treated with a constant infusion of 18 pmol/24 h of EB1089.

After tumor cells implantation, a progressive increase in tumor volume was observed that preceded an increase in plasma calcium (Fig. 1). Blood was collected at timed intervals by orbital bleeding, and total plasma calcium was measured by microchemistry. Results represent the mean ± SE of eight animals in each group. Animals were treated at time 0 with vehicle alone (ΔC) or with EB1089 (●). *, significant difference (P < 0.01) in plasma calcium from control tumor-bearing animals (vehicle alone) at the time points indicated (+3, +15). †, significant change (P < 0.01) from plasma calcium at time 0 (pretreatment).

Table 2 Effect of EB1089 on FBS-stimulated cell growth in HPK1A-ras cells in vitro and on tumor growth in vivo.

<table>
<thead>
<tr>
<th>EB1089 concentration (nm)</th>
<th>Cell number as % control</th>
<th>Formazan production as % control</th>
<th>Tumor growth as % increase of pretreatment volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (100%)</td>
<td>95 ± 32</td>
<td>92.3 ± 3.2 Wouldn't you prefer</td>
<td>16.8 ± 5.0 Would you like it</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>86.4 ± 3.5</td>
<td>92.3 ± 3.2</td>
<td>16.8 ± 5.0</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>82.4 ± 0.45</td>
<td>78.3 ± 5.0</td>
<td>16.8 ± 5.0</td>
</tr>
</tbody>
</table>

* Significant difference from control values or vehicle-treated animals (P < 0.05).
clinical syndrome of MAH. Our strategy was to use a continuous infusion of EB1089, which does not produce calcium elevation in control non-tumor-bearing animals. A dosage of 18 pmol/24 h was determined, and the pump was implanted adjacent to the tumor to deliver a high concentration of the analogue to the tumor site. This experimental design was favored to achieve maximal local inhibition of PTHRP production by tumor cells. Although we cannot exclude a strong systemic effect of EB1089, it is likely that this experimental design favors a strong local effect of EB1089 on the tumor. To be useful clinically, such agents will require adequate modes of delivery in cancer patients to mimic the experimental design presented here.

The administration of EB1089 was effective in reversing hypercalcemia in tumor-bearing animals in which calcium levels were ≥2.85 mmol/liter. This cutoff value was used because it represents the clinical situation in cancer patients in which hypercalcemia often requires treatment with antiresorptive agents such as bisphosphonates. Bisphosphonates are highly effective in reversing hypercalcemia but their effect are short-lived (29), and patients with elevated PTHRP levels are often resistant to these agents (30). Consequently, an agent that inhibits PTHRP production would represent a major advance in the treatment of this common condition. Our study clearly indicates the EB1089 blocks PTHRP production and decreases its levels to near normal values. Infusion of the analogue also significantly reduced the growth velocity of the tumor-bearing animals. A dosage of 18 pmol/24 h was determined, and the pump was implanted adjacent to the tumor to deliver a high concentration of the analogue to the tumor site. This experimental design was favored to achieve maximal local inhibition of PTHRP production by tumor cells. Although we cannot exclude a strong systemic effect of EB1089, it is likely that this experimental design favors a strong local effect of EB1089 on the tumor. To be useful clinically, such agents will require adequate modes of delivery in cancer patients to mimic the experimental design presented here.

The mechanism(s) by which 1,25(OH)2D3 and its analogues inhibit cell growth remains elusive. One postulated mechanism is that they modulate the expression of cell-cycle-associated genes. We and others have shown that 1,25(OH)2D3 inhibit the expression of the c-myc oncogene (32–34) and also blocks the progression from G0-G1 to the S phase of the cell cycle (35, 36). Another potential mechanism is that EB1089 works independently via the inhibition of PTHRP. Indeed, PTHRP purification of plasma calcium reduction was not a direct result of tumor shrinkage. Our in vitro data indicate that the effect of EB1089 seen on tumor growth is secondary to a direct effect of the vitamin D analogue on cellular proliferation as previously reported for other cell types (26, 31). In vitro survival assays indicate that the effect of EB1089 is unlikely to be secondary to apoptotic cell death and correlates well with the absence of an in vivo effect on tumor shrinkage.

Acknowledgments

We thank Lise Bindere (Leo Pharmaceuticals Ltd., Ballerup, Denmark) for providing EB1089 and Pamela Kirk for preparation of the manuscript.
Reversal of Hypercalcemia with the Vitamin D Analogue EB1089 in a Human Model of Squamous Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/14/3325

Cited articles
This article cites 31 articles, 5 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/14/3325.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/59/14/3325.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.