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Research Article

We have examined the effects of 1,25 dihydroxyvitamin D3 (1,25[OH]2D3) and a low calcemic analogue EB1089 on parathyroid hormone-related peptide (PTHrP) production and on the development of hypercalcemia in Fischer rats implanted with the Leydig cell tumor H-500. Leydig cell tumors were implanted subcutaneously into male Fischer rats, which received constant infusions intraperitoneally of either 1,25(OH)2D3 (50-200 pmol/24 h), EB1089 (50-400 pmol/24 h), or vehicle for up to 4 wk. A control group of animals received similar infusions without tumor implantation. Plasma calcium, plasma levels of immunoreactive iPTHrP, and tumor PTHrP mRNA levels were determined as well as tumor size, animal body weight, and animal survival time. Non-tumor-bearing animals receiving > 50 pmol/24 h of 1,25(OH)2D3 became hypercalcemic, whereas no significant change in plasma calcium was observed in animals receiving < or = 200 pmol/24 h of EB1089. Tumor-bearing animals receiving vehicle alone or > 50 pmol/24 h of 1,25(OH)2D3 became severely hypercalcemic within 15 d. However, animals treated with low dose 1,25(OH)2D3 and all doses of EB1089 maintained near-normal or normal levels of plasma calcium for up to 4 wk. Additionally, reduced levels of tumor PTHrP mRNA and of plasma iPTHrP were observed compared with controls in both vitamin D- and EB1089-treated rats. Infusion of 50 pmol/24 h of 1,25(OH)2D3 and 200 pmol/24 h of EB1089 significantly reduced tumor [...]

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A Vitamin D Analogue (EB1089) Inhibits Parathyroid Hormone-related Peptide Production and Prevents the Development of Malignancy-associated Hypercalcemia In Vivo

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Abstract

We have examined the effects of 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) and a low calcemic analogue EB1089 on parathyroid hormone-related peptide (PTHrP) production and on the development of hypercalcemia in Fischer rats implanted with the Leydig cell tumor H-500. Leydig cell tumors were implanted subcutaneously into male Fischer rats, which received constant infusions intraperitoneally of either 1,25(OH)₂D₃ (50–200 pmol/24 h), EB1089 (50–400 pmol/24 h), or vehicle for up to 4 wk. A control group of animals received similar infusions without tumor implantation. Plasma calcium, plasma levels of immunoreactive iPTHrP, and tumor PTHrP mRNA levels were determined as well as tumor size, animal body weight, and animal survival time. Non-tumor-bearing animals receiving >50 pmol/24 h of 1,25(OH)₂D₃ became hypercalcemic, whereas no significant change in plasma calcium was observed in animals receiving ≤200 pmol/24 h of EB1089. Tumor-bearing animals receiving vehicle alone or >50 pmol/24 h of 1,25(OH)₂D₃ became severely hypercalcemic within 15 d. However, animals treated with low dose 1,25(OH)₂D₃ and all doses of EB1089 maintained near-normal or normal levels of plasma calcium for up to 4 wk. Additionally, reduced levels of tumor PTHrP mRNA and of plasma iPTHrP were observed compared with controls in both vitamin D- and EB1089-treated rats. Infusion of 50 pmol/24 h of 1,25(OH)₂D₃ and 200 pmol/24 h of EB1089 significantly reduced tumor volume by the end of experiment. The analogue but not 1,25(OH)₂D₃ substantially prolonged survival time in tumor-bearing animals with longer survival achieved at the highest dose, 400 pmol/24 h, of EB1089. These studies demonstrate that 1,25(OH)₂D₃ and a low calcemic vitamin D analogue are potent inhibitors of PTHrP production *in vivo*. Low calcemic analogues may therefore represent important alternative therapy for malignancy-associated hypercalcemia. (*J. Clin. Invest.* 1993. 91:2416–2422.) Key words: vitamin D • parathyroid hormone-related peptide • malignancy • hypercalcemia • Leydig cell tumor

Introduction

Hypercalcemia is a common complication of human malignancy and may produce considerable morbidity and mortality.

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Parathyroid hormone-related peptide (PTHrP)¹ is a recently described entity that has been shown to be a pathogenetic mediator of humoral hypercalcemia of malignancy (1–6). Hypercalcemia may also occur in association with a number of animal neoplasms. Among the best characterized of the animal models, and one which closely mimics the human syndrome, is the male Fischer rat implanted with the Rice 500 Leydig cell tumor (7, 8). PTHrP is well documented to cause the hypercalcemia associated with this tumor (9, 10). Currently, the mainstays of therapy for malignancy-associated hypercalcemia are agents that inhibit skeletal calcium mobilization, such as bisphosphonates and calcitonin, or agents that enhance renal calcium clearance, such as loop diuretics. However, PTHrP is known to cause hypercalcemia by its action on both bone and kidney. Consequently, a therapeutic strategy aimed at inhibiting PTHrP production and thereby neutralizing both mechanisms simultaneously might be a useful addition to our antihypercalcemic armamentarium. To date, no such strategy has been reported.

PTHrP expression has been shown to be regulated by multiple factors in a variety of human cell lines *in vitro*, including rat Leydig cells grown in culture. We have recently shown that in cultured Leydig cells several steroidal factors such as testosterone and dexamethasone inhibit PTHrP gene expression and secretion. The seco-steroid 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) is also inhibitory of PTHrP production *in vitro* both in this tumor and in other normal and malignant cell types (11–14). Nevertheless the *in vivo* efficacy of this substance is unknown. In the present study we have therefore examined the capacity of 1,25(OH)₂D₃ and of a low calcemic vitamin D analogue, EB1089, to inhibit PTHrP production by the Rice 500 Leydig cell tumor *in vivo* and to prevent the development of hypercalcemia. Effects on body weight and tumor volume were also examined and the effect on animal survival time was assessed.

Methods

Vitamin D and analogue. 1,25-(OH)₂D₃ was a generous gift of Dr. M. Uskokovic of Roche Pharmaceuticals (Nutley, NJ). EB1089 is an analogue of 1,25(OH)₂D₃ which was kindly provided by Leo Pharmaceuticals (Ballerup, Denmark) (15).

Animal protocols. Male Fischer 344 rats (Charles River Breeding Laboratories, Inc., Wilmington, MA), weighing 200–220 g (10 wk of age), were used in all studies. Animals were housed individually and maintained on tap water ad lib. and standard rodent chow (rat chow

1. Abbreviations used in this paper: iPTHrP, immunoreactive parathyroid hormone-related peptide; 1,25(OH)₂D₃, 1,25 dihydroxyvitamin D₃; PTHrP, parathyroid hormone-related peptide.

5012; Ralston-Purina Canada, Inc., Lasalle, Quebec, Canada) containing 1% calcium and 0.74% phosphorus (16). The Rice-500 Leydig cell tumor was obtained from the National Cancer Institute Breast Cancer Task Force Bank (EEG Mason Research Institute, Worcester, MA). The tumor was minced in normal saline to give a suspension of one part of tumor to one part of saline. Tumor suspension (0.2 ml) was then injected into the right flank of each test animal. Control animals were weight matched and maintained under identical conditions (9).

To examine the effect of 1,25(OH)₂D₃ and EB1089 on tumor-bearing animals, osmotic minipumps (model 2ML4 Alzet; Alza Corporation, Palo Alto, CA) were implanted intraperitoneally under light ether anesthesia. Each minipump contained either 1,25(OH)₂D₃ or EB1089 dissolved in 1 ml of propylene glycol, 200 μl of ethanol, and 800 μl of saline to deliver a continuous dose of the compound for up to 4 wk at a delivery rate of 2.5 μl/h (17). On the day of tumor implantation all tumor-bearing animals also received a loading dose equivalent to one daily dose of either 1,25(OH)₂D₃ or EB1089. One group of tumor-bearing animals received vehicle alone as a control. Other control groups of sham-operated animals without tumor received either 1,25(OH)₂D₃, EB1089, or vehicle alone through osmotic minipumps as described above for up to 4 wk.

All animals were weighed every third day for up to 4 wk. Blood samples were collected every third day from each group under ether anesthesia and plasma samples were stored at -20°C until the time of analysis. The tumor mass of control and experimental animals was measured in two dimensions by calipers and the tumor volume was calculated (18). Tumor tissue was also extracted for RNA analysis.

Analytical methods. Plasma calcium levels were determined by atomic-absorption spectrophotometry (model 703; Perkin-Elmer Corp., Norwalk, CT).

For RIA of PTHRP, rat plasma was tested in two dilutions in duplicate using an NH₂-terminal RIA, as described previously (19), and values were assigned using a PTHRP(1-34) standard. Consequently, all values are in picomole equivalents per liter relative to PTHRP(1-34). These samples were also tested in an NH₂-terminal RIA kit (Incstar Corporation, Stillwater, MN), according to manufacturer's instructions.

For Northern blot analysis, 20 μg of total RNA was electrophoresed on a 1.1% agarose-formaldehyde gel and transferred by blotting to a nylon membrane (20). The filters were hybridized (21) with an EcoRI, XbaI restriction fragment of PTHRP cDNA M-9 and a rat cyclophilin 800-bp pair BamHI restriction fragment from plasmid pCD15.8.1 (6, 22). Each was labeled with [³²P]dCTP by the random primer method (23). After incubation at 42°C for 24 h, filters were washed twice for 30 min each in 2× SSC, 0.1% SDS at 50°C (1× SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate). Autoradiography of filters was then carried out and the intensity of the autoradiograms was analyzed by laser densitometry (Ultrosan XL; LKB Instruments Inc., Gaithersburg, MD). The signals for PTHRP mRNA were related to that for cyclophilin mRNA, which was not changed by the various agents tested.

Statistical analysis. All results are expressed as the mean±SEM, and statistical comparisons are made on the basis of a Student's *t* test or a one-way analysis of variance, using a Bonferroni adjustment where appropriate (24).

Results

Effects on hypercalcemia

Effect of 1,25(OH)₂D₃ and EB1089 on plasma calcium. In non-tumor-bearing animals the lowest dose of 1,25(OH)₂D₃ (50 pmol/24 h) did not produce significant hypercalcemia, however, higher doses produced a progressive increase in plasma calcium (Fig. 1, top). In contrast, non-tumor-bearing animals receiving up to 200 pmol/24 h of EB1089 did not show any

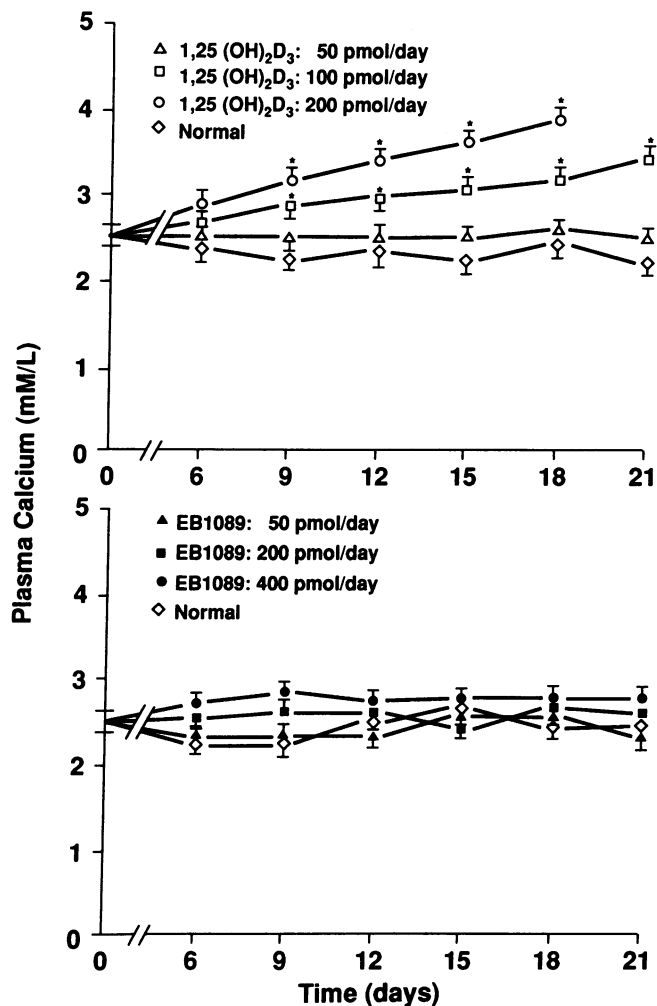


Figure 1. Effect of 1,25(OH)₂D₃ and EB1089 on plasma calcium in normal rats. Normal non-tumor-bearing male Fischer rats were infused with 50, 100, or 200 pmol/24 h of 1,25(OH)₂D₃ or with vehicle alone as described in Methods (top). Another group of animals were infused with 50, 200, or 400 pmol/24 h of EB1089 or vehicle alone (bottom). Animals were killed at timed intervals and their plasma calcium was determined as described in Methods. Results represent the mean±SEM of six starting animals in each group in four different experiments. Significant differences in plasma calcium from control non-tumor-bearing animals at each time point are represented by asterisks (**P* < 0.05).

significant increase in plasma calcium when compared to the vehicle-treated control group (Fig. 1, bottom).

Control vehicle-treated tumor-bearing animals showed a progressive increase in their plasma calcium starting 12-days after implantation (Fig. 2). The rise in plasma calcium was more pronounced in the tumor-bearing animals receiving 100 pmol/24 h of 1,25(OH)₂D₃; those receiving 50 pmol/24 h of 1,25(OH)₂D₃ showed a significantly lower rise in plasma calcium as compared with the control animals (Fig. 2, top). Nevertheless, these animals did become hypercalcemic towards the end of experiment. In contrast, tumor-bearing animals infused with ≤200 pmol/24 h of the analogue maintained near-normal plasma levels comparable to the calcium levels of non-tumor-bearing rats (Fig. 2, bottom). This effect was sustained throughout the infusion of this vitamin D analogue (Fig. 2, bottom).

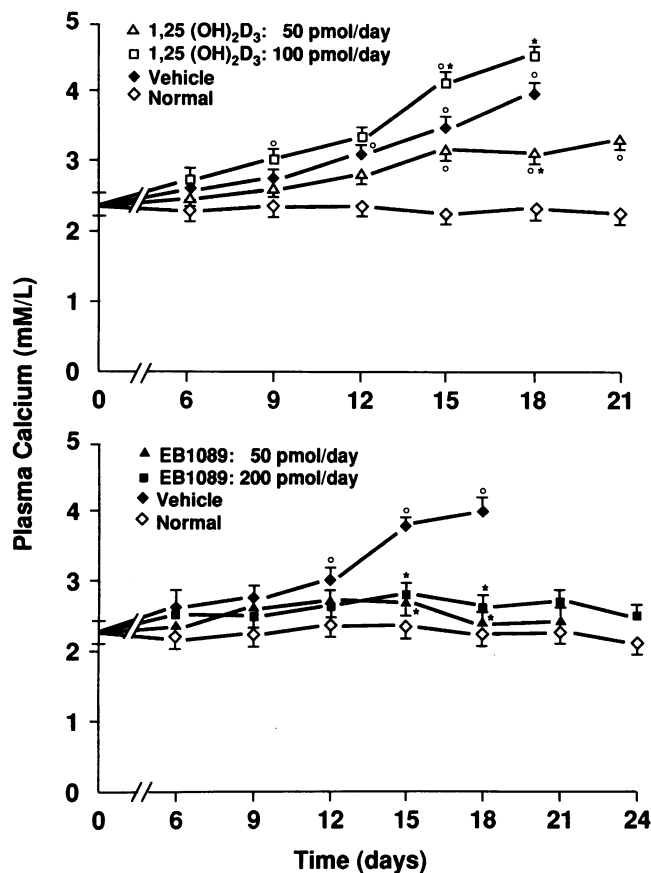


Figure 2. Effect of 1,25(OH)₂D₃ and EB1089 on plasma calcium in H-500 tumor-bearing rats. Tumor-bearing male Fischer rats were infused with 50 or 100 pmol/24 h of 1,25(OH)₂D₃, or with vehicle alone as described in Methods (*top*). Another group of animals was infused with 50 or 200 pmol/24 h of EB1089 or with vehicle alone (*bottom*). Animals were killed at timed intervals and their plasma calcium was determined as described in Methods. Plasma calcium in normal non-tumor-bearing animals is also shown. Results represent the mean±SEM of six starting animals in each group in four different experiments. Significant differences in plasma calcium from control tumor-bearing and normal non-tumor-bearing animals at each time point are represented by * and ○, respectively (*,○ *P* < 0.05).

Effects on tumor characteristics

Effect on animal weight. Normal vehicle-treated rats without tumor implantation showed a progressive gain in weight during the study (Fig. 3). Animals receiving a low dose of 1,25(OH)₂D₃ also displayed a gain in body weight, however, the higher doses induced a drop in body weight (Fig. 3, *top*). In contrast all doses of EB1089 permitted a progressive gain in weight over time that was parallel to the vehicle-treated control group in these non-tumor-bearing rats (Fig. 3, *bottom*).

Tumor-bearing animals receiving vehicle alone progressively decreased their weight during the study, and at day 18 after tumor implantation, all animals in this group died (Fig. 4). Animals receiving 100 pmol/24 h of 1,25(OH)₂D₃ showed a more pronounced body weight decline than the control group, whereas the animals receiving 50 pmol/24 h of 1,25(OH)₂D₃ had a less dramatic drop in body weight, which was noticeable only by day 18 (Fig. 4, *top*). In contrast, all tumor-

bearing animals receiving the different doses of EB1089 exhibited a gain in weight up to day 15 and then slowly decreased their weight. A more marked drop was observed with the lowest dose (50 pmol/24 h) of EB1089 (Fig. 4, *bottom*).

Effect on tumor volume. Tumor-bearing animals receiving vehicle alone showed a progressive increase in their tumor volume from day 12 until their death by day 18 (Fig. 5). Animals receiving 50 pmol/24 h of 1,25(OH)₂D₃ or 200 pmol/24 h of EB1089 showed no difference in their tumor volume up to day 15 compared with control tumor-bearing animals receiving vehicle alone. However, by day 18 after tumor implantation, the tumor volume of the animals treated with 1,25(OH)₂D₃ or EB1089 was significantly reduced. Furthermore, animals receiving these agents survived beyond day 18 and did not exhibit any further increase in their tumor volume (data not shown).

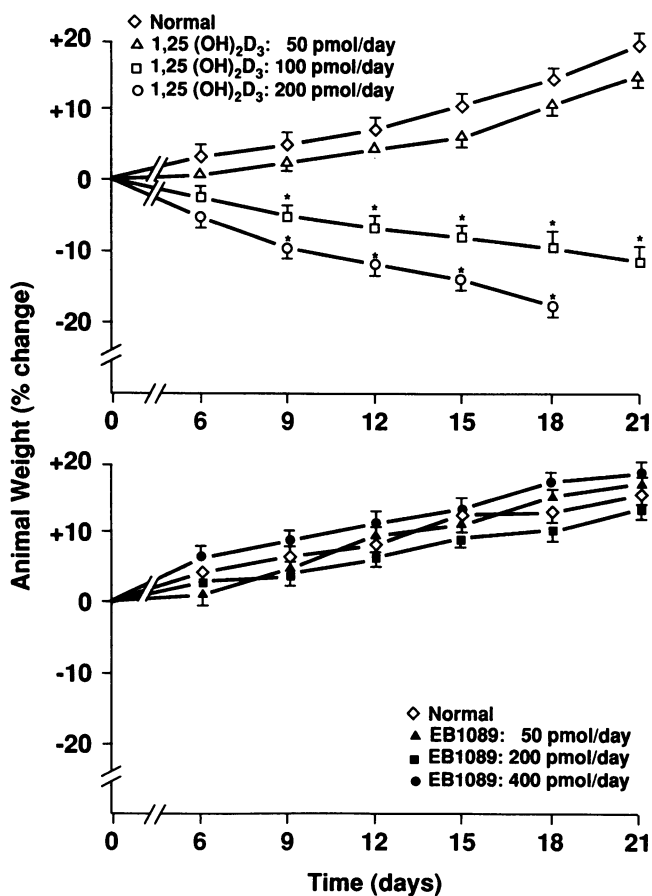


Figure 3. Effect of 1,25(OH)₂D₃ and EB1089 on body weight in normal animals. Normal non-tumor-bearing male Fischer rats were infused with 50, 100, or 200 pmol/24 h of 1,25(OH)₂D₃ or with vehicle alone as described in Methods (*top*). Another group of animals were infused with 50, 200, or 400 pmol/24 h of EB1089 or with vehicle alone (*bottom*). Animals were weighed at timed intervals and compared with their initial weight at the start of experiment. Results are expressed as percent change. Each time point represents the mean±SEM of six starting animals in each group in four different experiments. Significant differences in weight from control non-tumor-bearing animals at each time point are represented by asterisks (**P* < 0.01).

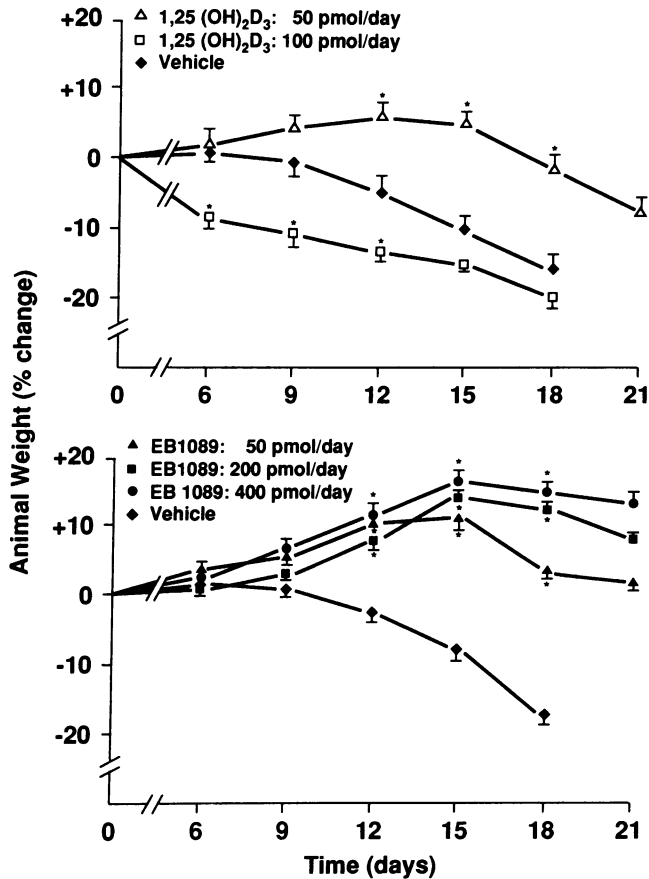


Figure 4. Effect of 1,25(OH)₂D₃ and EB1089 on body weight in H-500 tumor-bearing animals. Tumor-bearing male Fischer rats were infused with 50, 100 pmol/24 h of 1,25(OH)₂D₃, or with vehicle alone as described in Methods (*top*). Another group of animals were infused with 50, 200, or 400 pmol/24 h of EB1089 or with vehicle alone (*bottom*). Animals were weighed at timed intervals and compared with their own initial weight at the start of experiment. Results are expressed as percentage change. Each time point represents the mean±SEM of six starting animals in each group in four different experiments. Significant differences in weight from control tumor-bearing animals at each time point are represented by asterisks (**P* < 0.01).

Effects of 1,25(OH)₂D₃ and EB1089 on PTHRP production. PTHRP mRNA from the tumors of implanted animals was measured at timed intervals (days 12, 15, and 18). Tumor PTHRP mRNA increased over time in the animals receiving vehicle alone (Fig. 6). Animals receiving either 1,25(OH)₂D₃ (50 pmol/24 h) or its analogue EB1089 (200 pmol/24 h) demonstrated a dramatic decrease in PTHRP mRNA expression in tumor tissue compared with the vehicle-treated control group (Fig. 6). Similar results were seen with 100 pmol/24 h of 1,25(OH)₂D₃.

Immunoreactive PTHRP (iPTHRP) was undetectable in non-tumor-bearing animals. However, all tumor-bearing animals had detectable concentrations of plasma iPTHRP as of 12 d after tumor implantation and the levels progressively increased (Fig. 7). Comparison of PTHRP immunoreactivity at days 12, 15, and 18 among the various treatment groups re-

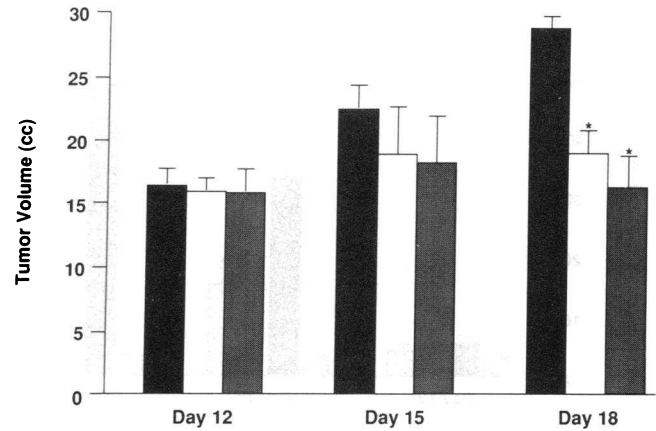


Figure 5. Effect of 1,25(OH)₂D₃ and EB1089 on tumor volume. Tumor volume was determined at timed intervals as described in Methods. Tumor volume after treatment of animals with 50 pmol/24 h 1,25(OH)₂D₃ (□) or 200 pmol/24 h EB1089 (■) was compared with the volume of tumors in animals receiving the vehicle alone (■). Results represent the mean±SEM of six starting animals in each group in four different experiments. Significant differences from control tumor-bearing animals at each time point are represented by asterisks (**P* < 0.05).

vealed a decrease in iPTHRP concentrations in animals receiving either 1,25(OH)₂D₃ or its analogue EB1089 compared with the tumor-bearing animals receiving vehicle alone (Fig. 7).

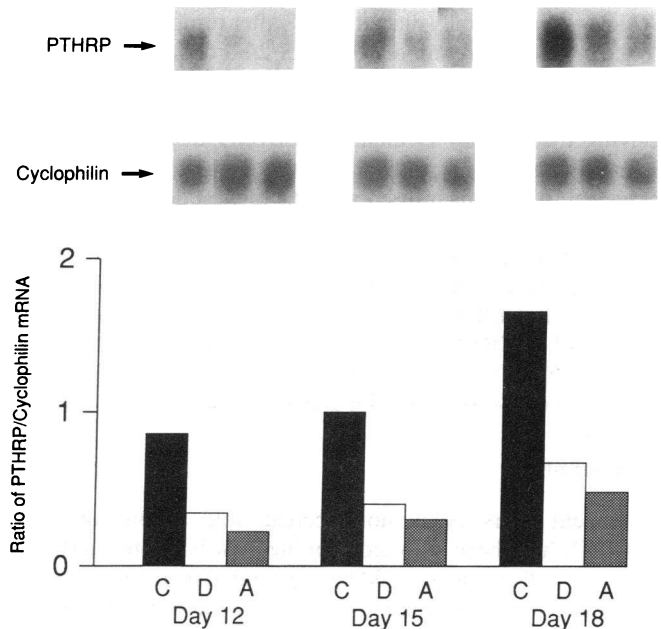


Figure 6. Effect of 1,25(OH)₂D₃ and EB1089 on PTHRP mRNA expression in H-500 tumors. PTHRP mRNA expression was determined in tumor extracts, as described in Methods, at timed intervals after the onset of tumor growth. PTHRP mRNA expression (*top*) after treatment of animals with 50 pmol/24 h 1,25(OH)₂D₃ (D) or 200 pmol/24 h of the analogue EB1089 (A) was compared with PTHRP mRNA expression in control tumors from animals receiving the vehicle alone (C). All blots were quantified by probing with cyclophilin as a control (*bottom*). Ratios of PTHRP/cyclophilin mRNA are shown in the figure below the panels.

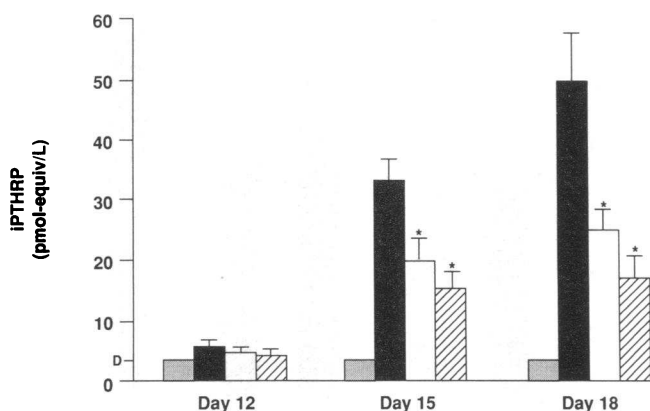


Figure 7. Effect of 1,25(OH)₂D₃ and EB1089 on plasma iPTHrP concentrations in H-500 tumor-bearing animals. PTHrP concentrations were determined as described in Methods at timed intervals. PTHrP concentrations were examined in non-tumor-bearing animals receiving vehicle alone (□) and in H-500 tumor-bearing animals receiving vehicle alone (■), 50 pmol/24 h of 1,25(OH)₂D₃ (▨) or 200 pmol/24 h of EB1089 (▩). *D* represents the detection limit of the assay. Results represent the mean ± SEM of six starting animals in each group in four different experiments. Significant differences from control tumor-bearing animals at each time point are represented by asterisks (**P* < 0.01).

Effect of EB1089 on survival time. We next assessed the effect of either 1,25(OH)₂D₃ or its analogue EB1089 on survival time of the tumor-bearing animals. Animals receiving vehicle alone generally died at day 18 after tumor implantation (Fig. 8). Administration of 100 pmol/24 h of 1,25(OH)₂D₃ reduced the survival time whereas 50 pmol/24 h of 1,25(OH)₂D₃ produced a modest increase in survival. However, animals receiving the vitamin D analogue EB1089 displayed a more marked increase in survival time with the longest survival achieved in animals infused with the highest dose (400 pmol/24 h) of EB1089. By day 20 after tumor implantation, few of the animals receiving the low dose of 1,25(OH)₂D₃ (50 pmol/24 h) survived whereas 100% of the animals receiving 400 pmol/24 h of EB1089 and 84% of the animals receiving 200 pmol/24 h of EB1089 survived. By day 24, at which time none of the animals in the other groups were alive, 67% of the animals receiving 400 pmol/24 h of EB1089 had survived (Fig. 8).

Discussion

In recent years novel nonhypercalcemic actions of 1,25(OH)₂D₃ have been reported. Among these is a potent antiproliferative effect, which could be of potential therapeutic benefit in cancer states (25–27). However, the inherent hypercalcemic activity of 1,25(OH)₂D₃ has prevented its use as a potential therapeutic agent in this regard. Recently, intense efforts have resulted in the development of several 1,25(OH)₂D₃ analogues that exhibit low calcemic activity. The major modifications of 1,25(OH)₂D₃ that have resulted in low calcemic analogues have been structural changes in the side chain, such as introduction of oxygen atoms and stereochemical changes (27–29). We have used one such analogue, EB1089, in our studies. In this analogue the side chain has been elongated with introduction of terminal ethyl groups and double bonds have been intro-

duced at positions 22 and 24 (16). Low calcemic analogues of vitamin D retain the antiproliferative effects of 1,25(OH)₂D₃ but are far less active in absorption of intestinal calcium and mobilization of calcium from bone (28). Indeed EB1089 has previously been reported to be ~ 60 times more active in inhibiting cell proliferation and inducing cell differentiation than 1,25(OH)₂D₃ in U937 histiocytic lymphoma cells *in vitro* while having a threefold lower calcemic activity in normal rats *in vivo* (30). In addition, in common with 1,25(OH)₂D₃, several of the low calcemic analogues may decrease the synthesis of parathyroid hormone, induce osteocalcin gene expression in bone cells, inhibit angiogenesis, and display immunoregulatory activity (29, 31–33). Administration of such 1,25(OH)₂D₃ analogues either orally, intraperitoneally, or by direct injection into tumor tissue has been shown in certain models to decrease tumor volume and prolong animal survival without causing hypercalcemia (27, 28, 34).

We compared the capacity of 1,25(OH)₂D₃ and of EB1089 to control the syndrome of malignancy-associated hypercalcemia *in vivo*. The rationale of this approach was primarily made on the basis of our previous demonstration that both 1,25(OH)₂D₃ and calcipotriol, an analogue of 1,25(OH)₂D₃ with only low calcemic potency, could effectively inhibit PTHrP gene expression and PTHrP secretion from cells maintained in culture (35). Thus, the combination of the known antiproliferative actions of vitamin D and its analogues and its potent action on inhibiting PTHrP production suggested that it might be successfully employed in controlling malignancy-associated hypercalcemia *in vivo*. In view of the fact that calcipotriol did not appear to be a likely candidate for these studies because of its very short half-life, we used EB1089, which has recently been reported to have approximately the same half-life *in vivo* as 1,25(OH)₂D₃ (30).

In our studies EB1089 failed to significantly raise plasma calcium concentrations above normal at doses as high as eight times the highest dose of 1,25(OH)₂D₃ (50 pmol/24 h), which produced only mild hypercalcemia. Non-tumor-bearing animals remained healthy and indistinguishable from animals in-

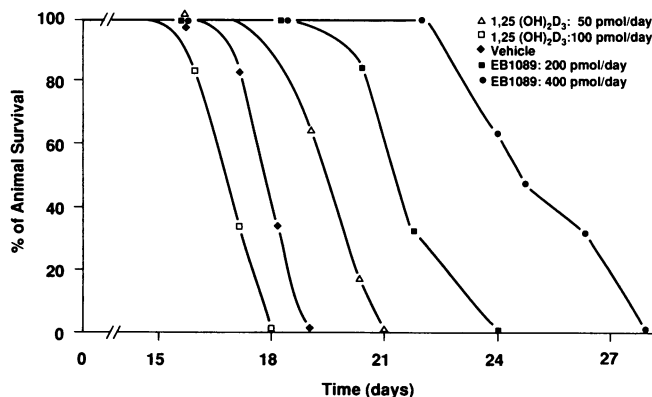


Figure 8. Effect of 1,25(OH)₂D₃ and EB1089 on animal survival time. The number of surviving tumor-bearing animals was assessed after infusion with vehicle alone, with 1,25(OH)₂D₃ at a dose of 50 or 100 pmol/24 h, or with EB1089 at a dose of 200 pmol/24 h or 400 pmol/24 h. Each group consisted of six animals and percentage survival at each time point was calculated.

fused with vehicle alone for the duration of the experiment. These results predicted that EB1089 could be used safely in the cancer state and could be used at substantially higher doses than $1,25(\text{OH})_2\text{D}_3$ to enhance its potential antitumor effects in our model. Administration of the analogue to tumor-bearing animals, even in the lowest dose used, was effective in preventing overt hypercalcemia. The lowest dose of $1,25(\text{OH})_2\text{D}_3$ used was almost as effective, but doubling of this dose, and further increases, resulted in rapidly progressing hypercalcemia and death of the animals. This hypercalcemia occurred despite inhibition of PTHRP production, indicating that this was due to the intrinsic actions of $1,25(\text{OH})_2\text{D}_3$. In previous studies it has been reported that infusions of 7 pmol/24 h of $1,25(\text{OH})_2\text{D}_3$ are required to restore normocalcemia and normal plasma $1,25(\text{OH})_2\text{D}_3$ concentrations in hypocalcemic vitamin D-depleted rats (36). In our study normal animals received Leydig cell tumor implants and in these animals normal plasma concentrations of $1,25(\text{OH})_2\text{D}_3$ were incapable of inhibiting PTHRP production or hypercalcemia. Consequently, it appears likely that only infusions between 7 and 50 pmol/24 h of $1,25(\text{OH})_2\text{D}_3$ would be effective in reducing tumor induced hypercalcemia. Whether the therapeutic window of $1,25(\text{OH})_2\text{D}_3$ is significantly smaller than that of the analogue EB1089 or of newer generations of such analogues will require further study but $1,25(\text{OH})_2\text{D}_3$ and EB1089 are clearly both effective *in vivo*.

Analysis of PTHRP mRNA levels in the tumor confirmed our hypothesis that $1,25(\text{OH})_2\text{D}_3$ and its analogue could inhibit PTHRP gene expression *in vivo* as well as *in vitro*. This inhibition was unlikely to be simply due to the antiproliferative effects of these agents, inasmuch as mRNA encoding PTHRP was selectively reduced relative to cyclophylin mRNA. *In vitro*, at least part of the inhibitory effect of vitamin D appears to be at the level of PTHRP gene transcription (11). Whether similar molecular mechanisms are involved in the *in vivo* inhibitory actions of vitamin D and its analogue remains to be determined. Circulating levels of iPTH_{RP} were also diminished by $1,25(\text{OH})_2\text{D}_3$ and its analogue in keeping with its inhibitory effect on PTHRP secretion *in vitro*. Although iPTH_{RP} concentrations were reduced as compared with control tumor-bearing animals, they were not completely suppressed. This nonsuppressible immunoreactivity may represent biologically inert NH₂-terminal material or may represent a level of biologically active PTHRP whose hypercalcemic activity may be neutralized by compensatory mechanisms. An analogous situation has been reported in humans with tumors in which elevated circulating concentrations of PTHRP have been described in association with normocalcemia (19, 37, 38).

We have also examined beneficial antineoplastic properties of $1,25(\text{OH})_2\text{D}_3$ and EB1089 other than inhibition of PTHRP production. A reduction in tumor volume was observed with both the lowest dose of $1,25(\text{OH})_2\text{D}_3$ used as well as with the higher dose of analogue used. Such a reduction may well have been due to the antiproliferative activity of both vitamin D and its analogue. However, even in the absence of severe hypercalcemia, the low dose of $1,25(\text{OH})_2\text{D}_3$ was unable to prevent weight loss after day 12 and survival time was markedly lower than with the higher concentrations of analogue that could be used. These results suggest that the analogue may have additional properties at the concentration used, such as the inhibition of other tumor products that contribute to cachexia and

death. Further studies will be required to evaluate these possibilities.

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