[CANCER RESEARCH 59, 3325–3328, July 15, 1999]

Advances in Brief

Reversal of Hypercalcemia with the Vitamin D Analogue EB1089 in a Human Model of Squamous Cancer[1]

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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 cm^3. 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 $1,25(OH)_2D_3$[3] 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 $1,25(OH)_2D_3$ is seriously limited by its side effects, which include hypercalcemia and hypercalciuria (7). Although its biological properties and its effect on tumor growth makes $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ requiring 10- to 100-fold higher concentrations of $1,25(OH)_2D_3$ to achieve the same effects (11, 12). To develop alternative strategies to block PTHRP production *in vitro* and *in vivo*, several $1,25(OH)_2D_3$ analogues—known to have low calcemic activities yet to retain strong antiproliferative 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 $1,25(OH)_2D_3$ yet is 10 times less potent in promoting hypercalcemia in rats (14). In the tumor progression model, EB1089 was 100 times more potent than $1,25(OH)_2D_3$ 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.

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[1] This work was supported by Medical Research Council of Canada Grant MT 10839 (to R. K.).

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[3] The abbreviations used are: $1,25(OH)_2D_3$, 1,25 dihydroxyvitamin D_3; PTHRP, parathyroid hormone-related peptide; MAH, malignancy-associated hypercalcemia; FBS, fetal bovine serum; iPTHRP, immunoreactive PTHRP.

Table 1 *Properties of 1,25(OH)$_2$D$_3$ and EB1089*

Compounds	Relative calcemic activity	*In vivo* half-life
1,25(OH)$_2$D$_3$	1	2.4 h[a]
EB1089	0.4[b]	2.8 h[a]

[a] Binderup *et al.* (14).
[b] Mathiasen *et al.* (22).

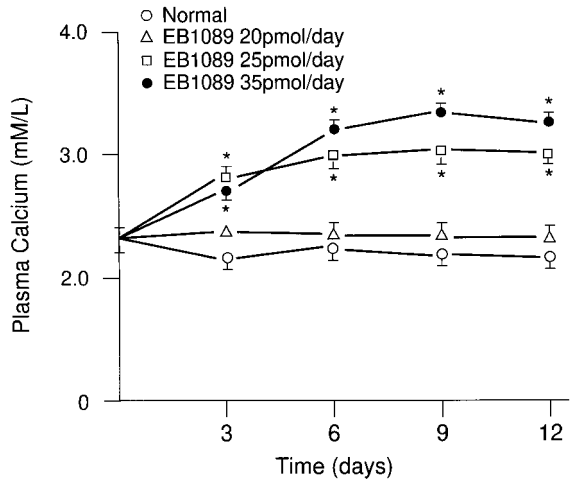

Fig. 1. Effect of EB1089 on plasma calcium in normal Balb C nude mice. Normal non-tumor-bearing animals were infused with 20, 25, or 35 pmol of EB1089 per 24 h or with vehicle alone (four animals per group) by constant infusion using Alzet osmotic minipumps. Blood was collected at timed intervals by orbital bleeding, and total plasma calcium was measured by microchemistry (Kodak Ektachem, Montreal, Quebec, Canada). Results represent the mean ± SE of three different experiments. *, significant difference ($P < 0.01$) in plasma calcium from control (vehicle alone) at each time point.

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 1×10^4 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 done as described previously (12). Cells were seeded at 2×10^3 cells/100 μl into 96-well microtiter plates using the same conditions as described above for cell growth. Formazan production was measured at 490 nm 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)$_2$D$_3$ *in vivo* (Ref. 22; Table 1).

Animal Protocols. BalbC nude mice (20 g; female) were implanted s.c. with 10^7 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:

$$\frac{4}{3}\Pi \times \left(\frac{L}{2}\frac{W}{2}\right)$$

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 Na_2HPO_4 (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 iPTHRP. 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).

Results

Effect of EB1089 on Plasma Calcium. In non-tumor-bearing animals the lowest dose of EB1089 (20 pmol/24 h) did not produce

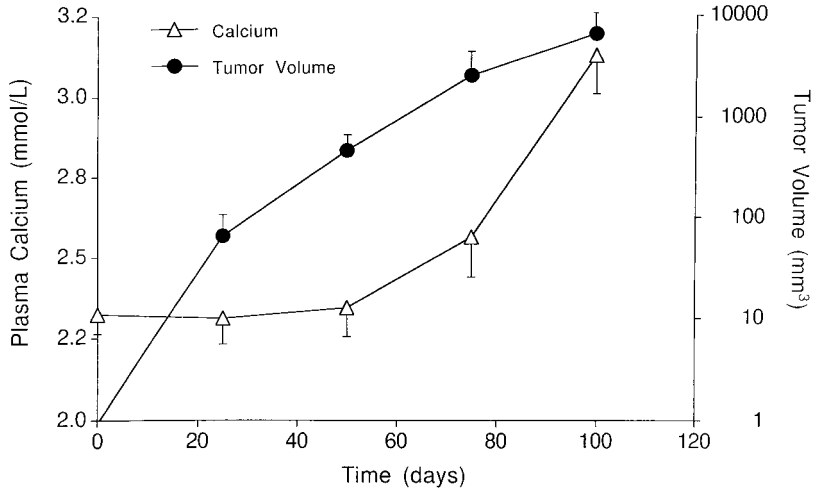

Fig. 2. Simultaneous analysis of tumor growth and plasma calcium. Balb C nude mice were implanted s.c. with 10^7 HPK1A-*ras* cells as described in "Materials and Methods." Tumor volume and plasma calcium were measured at timed intervals. Note that plasma calcium remains within the normal range until around 50 days after tumor implantation and starts to rise when the tumor size is above 500 mm^3.

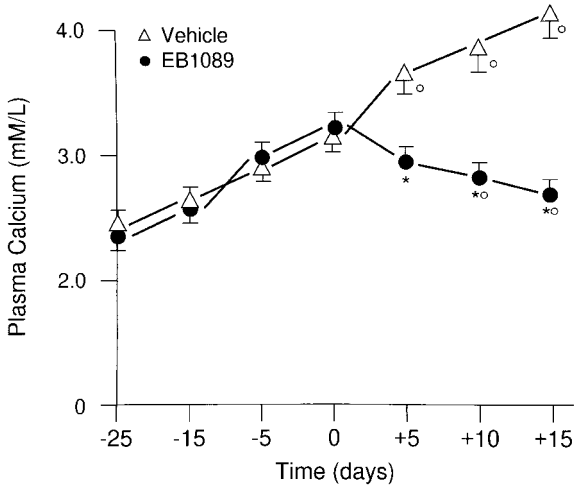

Fig. 3. Effect of EB1089 on plasma calcium in tumor-bearing animals. Balb C nude mice were infused at time 0 with 18 pmol of EB1089 per 24 h or with vehicle alone as described in Fig. 1. Blood was collected at timed intervals by orbital bleeding, and total plasma calcium was measured by microchemistry. Results represent the mean ± SE of eight animals in each group. Animals were treated at time 0 with vehicle alone (△-△) or with EB1089 (●-●). *, significant difference ($P < 0.01$) in plasma calcium from control tumor-bearing animals (vehicle alone) at the time points indicated (*+5, +15*). °, significant change ($P < 0.01$) from plasma calcium at time 0 (pretreatment).

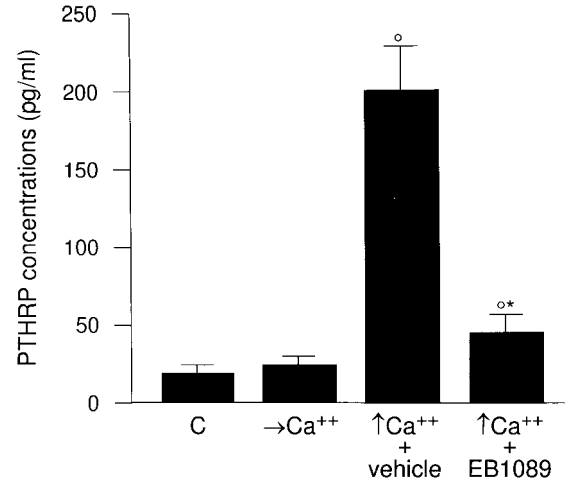

Fig. 4. Effect of EB1089 on plasma iPTHRP concentrations in tumor-bearing animals. Balb C nude mice were treated as described in Fig. 3. At the end of the infusion period (14 days), blood was collected by cardiac puncture, and plasma was kept at −70°C until assayed. PTHRP concentrations were determined using an immunoradiometric assay (DSL, Webster, Texas) specific for PTHRP 1–86. The detection limit of the assay is 2 pg/ml. Results represent the mean ± SE of eight animals in each group. *C*, controls, nontumor-bearing animals; $\rightarrow Ca^{++}$, normocalcemic tumor-bearing animals; $\uparrow Ca^{++}$, hypercalcemic tumor-bearing animals; *, significant difference ($P < 0.01$) from vehicle-treated animals; °, significant difference ($P < 0.01$) from C and $\uparrow Ca^{++}$.

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 starting between 6 and 10 weeks after tumor implantation (Fig. 2). A significant increase in plasma calcium occurred when tumor volume was above 0.5 cm^3. When plasma calcium was ≥2.85 mmol/liter, animals were implanted with Alzet osmotic minipumps and received EB1089 (18 pmol/24 h) or vehicle alone. Vehicle-treated animals showed a continuous increase in their plasma calcium (Fig. 3). In contrast, tumor- bearing animals—infused with the analogue (18 pmol/24 h)—demonstrated a significant decrease in their plasma calcium. Furthermore, these animals reached plasma calcium levels comparable to the calcium levels of non-tumor-bearing mice (Fig. 3).

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 cm^3 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

$1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$. 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

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

After 24 h in basal conditions (no FBS), fresh medium containing FBS (10%) and either 10^{-7} or 10^{-9} M EB1089 was added to cultures of HPK1A-*ras* cells for 72 h. Cell growth was assessed after cell trypsinization using a coulter counter. Formazan production was assessed using the XTT assay (12). Each value represents the mean ± SD of six determinations and is representative of three separate experiments. Tumor volume was assessed before and after the administration of EB1089 or vehicle alone and as a percentage increase of pretreatment tumor volume.

Cell proliferation			Tumor growth
EB1089 concentration (M)	Cell number % control	Formazan production	% increase of pretreatment size
Control (100%)			vehicle 95 ± 32
			EB1089 16.8 ± 5[a]
10^{-9}	86.4 ± 3.5[a]	92.3 ± 3.2[a]	
10^{-7}	82.4 ± 0.84[a]	78.3 ± 5.0[a]	

[a] 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 $\geq$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. However, tumor growth continued during treatment, and tumor volume was significantly higher after infusion than before EB1089 infusion indicating that 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.

The mechanism(s) by which $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ inhibit the expression of the c-*myc* oncogene (32–34) and also blocks the progression from G_0-G_1 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 was previously reported to promote tumor growth both *in vitro* and *in vivo* (37), and we cannot exclude an indirect effect of EB1089 on tumor growth via a reduction of PTHRP production by tumor cells.

Acknowledgments

We thank Lise Binderup (Leo Pharmaceuticals Ltd., Ballerup, Denmark) for providing EB1089 and Pamela Kirk for preparation of the manuscript.

References

1. Hosomi, J., Hosoi, J., Abe, E., Suda, T., and Kuroke, T. Regulation of terminal differentiation of cultured mouse epidermal cells by 1α, 25 dihydroxyvitamin D_3. Endocrinology, *113:* 1950–1957, 1983.
2. Smith, E. L., Walworth, N. C., and Holick, M. F. Effect of 1α, 25 dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. J. Invest. Dermatol., *86:* 709–714, 1986.
3. Colston, K. W., Colston, M. J., and Feldman, D. 1,25 dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology, *108:* 1083–1086, 1981.
4. Frampton, R. J., Omond, S. A., and Eisman, J. A. Inhibition of human cancer cell growth by 1,25 dihydroxyvitamin D_3. Cancer Res., *43:* 4443–4447, 1983.
5. Brehier, A., and Thomasset, M. Human colon cell line HT-29: characterization of 1,25 dihydroxyvitamin D_3 receptor and induction of differentiation by the hormone. J. Steroid Biochem. Mol. Biol., *29:* 265–270, 1988.
6. Eisman, J. A., Barkla, D. H., and Tutton, P. J. Suppression of *in vivo* growth of human cancer solid tumor xenographs by 1,25 dihydroxyvitamin D_3. Cancer Res., *47:* 21–25, 1987.
7. Bikkle, D. D. Clinical counterpoint: vitamin D: new actions, new analogs, new therapeutic potential. Endocr. Rev., *13:* 765–784, 1992.
8. Mundy, G. R., Ibbotson, K. J., D'Souza, S. M., Simpson, E. L., Jacobs, J. W., and Martin, T. J. The hypercalcemia of cancer. Clinical implications and pathogenic mechanisms. N. Engl. J. Med., *310:* 1718–1727, 1984.
9. Stewart, A. F., Horst, R., Deftos, L. J., Cadman, E. C., Lang, R., and Broadus, A. E. Biochemical evaluation of patients with cancer-associated hypercalcemia. Evidence for humoral and non-humoral groups. N. Engl. J. Med., *303:* 1377–1383, 1980.
10. Kremer, R., Karaplis, A. C., Henderson, J., Gulliver, W., Banville, D., Hendy, G. N., and Goltzman, D. Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes. J. Clin. Invest., *87:* 884–893, 1991.
11. Henderson, J., Goltzman, D., Sebag, M., Rhim, J., and Kremer, R. Dysregulation of parathyroid hormone-like peptide expression in a keratinocyte model of tumor cell progression. Cancer Res., *51:* 6521–6528, 1991.
12. Sebag, M., Henderson, J. E., Rhim, J., and Kremer, R. Relative resistance to 1,25 dihydroxyvitamin D_3 in a keratinocyte model of tumor progression. J. Biol. Chem. *267:* 12162–12167, 1992.
13. Yu, J., Papavasiliou, V., Rhim, J., Goltzman, D., and Kremer, R. Vitamin D analogs. New therapeutic agents for the treatment of squamous cancer and its associated hypercalcemia. Anticancer Drugs, *6:* 101–108, 1995.
14. Binderup, E., Calverley, M. J., and Binderup, L. Synthesis and biological activity of 1 α-hydroxylated vitamin D analogs with polyunsaturated side chains. *In:* A. W. Norman, R. Bouillon, and M. Thomasset (eds.), Vitamin D: Gene Regulation, Structure-Function Analysis and Clinical Application. Proceedings of the Eighth Workshop on Vitamin D, pp. 192–193. Berlin: de Gruyter, 1991.
15. Rice, B. F., Ponthier, R. L., and Miller, M. C. Hypercalcemia and neoplasia: a model system. Endocrinology, *88:* 1210–1216, 1971.
16. Rabbani, S. A., Mitchell, J., Roy, D. R., Kremer, R., Bennett, H. P. J., and Goltzman D. Purification of peptides with parathyroid hormone-like bioactivity from human and rat malignancies associated with hypercalcemia. Endocrinology, *118:* 1200–1210, 1986.
17. Henderson, J., Bernier, S., D'Amour, P., and Goltzman, D. Effects of passive immunization against parathyroid hormone (PTH)-like peptide and PTH in hypercalcemic tumor-bearing rats and normocalcemic controls. Endocrinology, *127:* 1310–1318, 1990.
18. Haq, M., Kremer, R., Goltzman, D., and Rabbani, S. A. A vitamin D analog (EB 1089) inhibits parathyroid hormone related peptide production and prevents the development of malignancy-associated hypercalcemia *in vivo*. J. Clin. Invest., *91:* 2416–2422, 1993.
19. Dürst, M., Dzarlieva-Petrusevska, R. T., Boukamp, P., Fusenig, N. E., and Gissman, L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene, *1:* 251–256, 1987.
20. Allen-Hoffmann, B. L., Sheibani, B., Hatfield, J. S., and Rhim, J. S. Mechanisms of carcinogenesis. *In:* Proceedings of the First Workshop on Neoplastic Transformation in Human Cell Systems *In Vitro*, pp. 25–34. New Jersey: Humana Press, 1991.
21. Dürst, M., Gallahan, D., Jay, G., and Rhim, J. S. Glucocorticoid-enhanced neoplastic transformation of human keratinocytes by human papillomavirus type 16 and an activated *ras* oncogene. Virology, *173:* 767–771, 1989.
22. Mathiasen, I. S., Colston, K. W., and Binderup, L. EB1089, a novel vitamin D analog, has strong antiproliferative and differentiation inducing effects on cancer cells. J. Steroid Biochem. Mol. Biol., *46:* 365–371, 1993.
23. Kremer, R., Shustik, C., Tabak, T., Papavasiliou, V., and Goltzman, D. Parathyroid hormone related peptide in hematologic malignancies. Am. J. Med., *100:* 406–411, 1996.
24. Glantz, S. A. Primer of Biostastics. New York: McGraw Hill, 1981.
25. Binderup, L., and Bramm, E. Effects of a novel vitamin D analog MC903 on cell proliferation and differentiation *in vitro* and on calcium metabolism *in vivo*. Biochem. Pharmacol., *37:* 889–894, 1988.
26. Colston, K. W., Chander, S. K., Mackay, A. G., and Coombes, R. C. Effects of synthetic vitamin D analogs on breast cancer cell proliferation *in vivo* and *in vitro*. Biochem. Pharmacol., *44:* 693–702, 1992.
27. Zhou, J-Y., Norman, A. W., Chen, D-L., Sun, G-W., Uskokovic, M., and Koeffler, H. P. 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 prolongs survival time of leukemic mice. Proc. Natl. Acad. Sci. USA, *87:* 3929–3932, 1990.
28. Abe, J., Nakano, T., Nishii, Y., Matsumoto, T., Ogata, E., and Ikeda, K. A novel vitamin D_3 analog, 22-oxa-1,25 dihydroxyvitamin D_3, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. Endocrinology, *129:* 832–837, 1991.
29. Fleisch, H. Bisphosphonates. Pharmacology and use in the treatment of tumor-induced hypercalcemia and metastatic bone disease. Drugs, *42:* 919–944, 1991.
30. Gurney, H., Grill, V., and Martin, T. J. Parathyroid hormone-related protein and response to pamidronate in tumour-induced hypercalcemia. Lancet, *341:* 1611–1613, 1993.
31. Colston, K. W., Mackay, A. G., James, S. Y., Binderup, L., Chander, S., and Coombes, R. C. EB1089: a new vitamin D analog which inhibits the growth of breast cancer cells *in vivo* and *in vitro*. Biochem. Pharmacol., *44:* 2273–2280, 1992.
32. Reitsma, P. H., Rothberg, P. G. Astrin, S. M., Trial, J., Bar-Shavit, Z., Hall, A., Teitelbaum, S. L., and Kahn, A. J. Regulation of *myc* oncogene expression in HL-60 leukemia cells by a vitamin D metabolite. Nature (Lond.), *306:* 492–494, 1983.
33. Kremer, R., Bolivar, I., Goltzman, D., and Hendy, G. N. Influence of calcium and 1,25 dihydroxyvitamin D_3 on proliferation and proto-oncogene expression in primary cultures of bovine parathyroid cells. Endocrinology, *125:* 935–942, 1989.
34. Matsumoto, K., Hashimoto, K., Nishida, Y., Hashiro, M., and Yoshikawa, K. Growth-inhibitory effects of 1,25 dihydroxyvitamin D_3 on human keratinocytes cultured in serum-free medium. Biochem. Biophys. Res. Commun., *166:* 916–923, 1990.
35. Sebag, M., Gulliver, W., and Kremer, R. Effects of 1,25 dihydroxyvitamin D_3 and calcium on growth and differentiation and on c-*fos* and *p53* gene expression in normal human keratinocytes. J. Invest. Dermatol., *103:* 323–329, 1994.
36. Rigby, W. F. C. The immunobiology of vitamin D. Immunol. Today, *9:* 54–58, 1998.
37. Rabbani, S. A., Gladu, J., Liu, B., and Goltzman, D. Regulation *in vivo* of the growth of Leydig cell tumors by antisense ribonucleic acid for parathyroid hormone-related peptide. Endocrinology, *136:* 5416–5422, 1995.