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Vitamin D analogs: new therapeutic agents for the treatment of squamous cancer and its associated hypercalcemia

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We have examined the *in vitro* effects of 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and of two side-chain modified analogs of 1,25$(OH)_2D_3$ (EB1089 and MC903) on cell growth and parathyroid hormone related peptide (PTHRP) production in immortalized (HPK1A) and neoplastic (HPK1A-*ras*) keratinocytes. Cell proliferation was strongly inhibited by 1,25$(OH)_2D_3$ and its analogs in HPK1A cells, and in this system EB1089 was 10–100 times more potent than 1,25$(OH)_2D_3$ or MC903. A similar effect on cell proliferation was observed in HPK1A-*ras* cells; however, 10-fold higher concentrations of 1,25$(OH)_2D_3$ or its analogs were required. We also observed a strong and dose-dependent inhibitory effect of these compounds on PTHRP secretion and gene expression. In both immortalized and neoplastic keratinocytes, EB1089 was 10–100 times more potent than 1,25$(OH)_2D_3$ or MC903 on inhibiting PTHRP production. However, although effective in HPK1A-*ras* cells, 10-fold higher concentrations of 1,25$(OH)_2D_3$ or its analogs were required to produce similar actions in this neoplastic model. These studies therefore demonstrate that a 1,25$(OH)_2D_3$ analog with low calcemic potency *in vivo* (EB1089) can inhibit keratinocyte proliferation and PTHRP production by such cells with greater potency than 1,25$(OH)_2D_3$. The observed effects of such analogs in neoplastic keratinocytes predicts their potential usefulness *in vivo* in inhibiting squamous cancer growth and its associated hypercalcemia.

Key words: Analogs, hypercalcemia, squamous cancer, vitamin D.

Introduction

Actions of 1,25 dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the most potent naturally occurring metabolite of vitamin D, are mediated through its vitamin D receptor, which is widely distributed in many tissues[1] including intestine, kidney, bone, hematopoietic cells, breast and skin. In addition to its classical effect on calcium homeostasis, 1,25$(OH)_2D_3$ has strong antiproliferative and prodifferentiative effects in several cell types.[2,3] In particular, addition of 1,25$(OH)_2D_3$ to cell cultures of keratinocytes inhibits proliferation and stimulates differentiation.[3] We have previously shown that 1,25$(OH)_2D_3$ is a potent inhibitor of parathyroid hormone related peptide (PTHRP) production in primary cultures of normal human keratinocytes *in vitro*[4] and that by analogy with other steroids the 1,25$(OH)_2D_3$ receptor complex interacts with *cis*-acting elements located in the promoter region of the gene.[5] However, 1,25$(OH)_2D_3$ administration *in vivo* is associated with side effects including hypercalcemia, hypercalciuria and bone resorption, and recent efforts have focused on obtaining potent analogs of 1,25$(OH)_2D_3$ devoid of these side effects. In the present study we have analyzed two of these compounds, with low *in vivo* calcemic activity, for their effects on cell growth and PTHRP production in immortalized and neoplastic keratinocytes cell lines *in vitro*. Calcipotriol (MC903), which has been used widely in the treatment of the keratinocyte proliferation seen in psoriasis,[6] was one of the analogs used. In this compound carbons at the 26 and 27 positions of the vitamin D side chain have been joined by a cyclopropyl ring and a hydroxyl group is at the 24 position (Figure 1). In addition there is a double bond at the 22–23 position. This analog was found to be 220 times less potent than 1,25$(OH)_2D_3$ in its ability to increase urinary and serum calcium and to decrease bone mass,[6] but has a very short half-life *in vivo*[7] (Table 1). EB1089, a more recently developed analog, was the other compound investigated. In this analog terminal ethyl groups and double bonds (at positions 22 and 24) have been introduced in the side chain (Figure 1). The compound has low cal-

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Table 1. Properties of $1,25(OH)_2D_3$, MC903 and EB1089

Compounds	Relative calcemic activity	*In vivo* half-life
$1,25(OH)_2D_3$	1	2.4 h[a]
MC903	0.005[b]	5 min[c]
EB1089	0.4[d]	2.8 h[a]

[a]Binderup *et al.*[8]
[b]Binderup *et al.*[6]
[c]Gumowski-Sunek *et al.*[7]
[d]Mathiasen *et al.*[9]

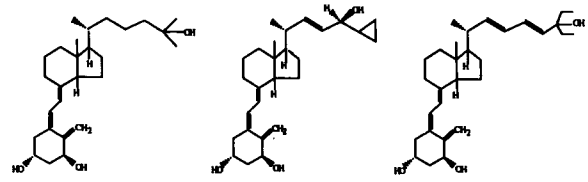

Figure 1. The structure of $1,25(OH)_2D_3$, MC903 and EB1089. MC903 and EB1089 differ only slightly in the side chain as compared with $1,25(OH)_2D_3$. EB1089 has two double bonds in the side chain and two ethyl groups instead of two methyl groups at the end of the side chain. MC903 has one double bond, one extra hydroxyl group and one cyclopropyl group at the end of the side chain.

cemic activity[8] and a half-life similar to $1,25(OH)_2D_3$ *in vivo*[9] (Table 1).

Materials and methods

Cell lines and culture conditions

Following transfection with HPV16 DNA, human keratinocytes in culture acquired an indefinite lifespan. These immortalized cells (HPK1A) retained many features characteristic of normal keratinocytes, such as contact inhibition, production of normal amounts of extracellular matrix proteins and development of cell-specific differentiation markers, when triggered to differentiate by loss of contact with substrata.[10] HPK1A cells were then neoplastically transformed into the malignant HPK1A-*ras* line following polybrene-induced transfer of pSV_2ras.[11] Transformation of these cells with an activated H-*ras* oncogene induced changes associated with the acquisition of a malignant phenotype, including loss of contact inhibition, colony formation in soft agar and production of invasive squamous cell carcinomas in nude mice.[12]

Cell lines were maintained as stocks in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with FBS (10%) (Gibco), 1 × antibiotic–antimycotic (Gibco) and passaged once or twice weekly. For all experiments cells were seeded in DMEM containing FBS (10%) at a density of 10^5 cells/well (10×10^3 cells/cm^2) for HPK1A and 10^5 cells/well (10×10^3 cells/cm^2) for HPK1A-*ras* in 6-well cluster plates. At 30% confluence (about 20×10^3 cells/cm^2), medium was replaced with fresh, unsupplemented DMEM (basal conditions) and incubation continued for 24 h. Following this 24 h serum-free period, medium was replaced with fresh unsupplemented DMEM or medium containing various combinations of 5% FBS, with or without $1,25(OH)_2D_3$ or its analogs. Incubations were continued at 37°C with 5% CO_2 for the appropriate times.

Following removal of conditioned medium, cells were trypsinized, dispersed and an aliquot taken for counting in a coulter counter (Coulter Elecronics, Bedfordshire, UK). Remaining cells were centrifuged at low speed, rinsed with PBS, lysed with 4 M guanidine thiocyanate, 25 mM trisodium citrate, 1 mM EDTA and 1 mM β-mercaptoethanol (GTC mix), and stored at −70°C for subsequent analysis by Northern blot hybridization.

Assay of immunoreactive PTHRP (iPTHRP) in conditioned medium

Conditioned medium, 1.5 ml/well, was removed at the appropriate times and centrifuged to remove debris. Duplicate aliquots of 200–500 µl were evaporated to dryness in a SPEEDVAC (Savant Instrument, Hicksville, NY) and stored at −20°C until assayed. Dried medium was reconstituted with 300 µl of outdated blood bank plasma (OBBP) and radioimmunoassayed as described previously.[4,11] Briefly, antiserum raised in rabbits against synthetic human (h) PTHRP(1–34) (Institut Armand Frappier, Montreal, Canada) was used at a final dilution of 1:35 000 and synthetic hPTHRP(1–34) diluted in OBBP was used as standard. The detection limit of the assay was 0.05 ng equivalents (ng-eq) of hPTHRP(1–34)/ml of conditioned medium. PTHRP immunoreactivity was undetectable in equivalent

amounts of unconditioned medium containing FBS (5%). Results were calculated as ng-eq of hPTHRP(1–34) corrected for cell number and expressed as percentage (%) of hPTHRP(1–34)/10^6 cells in the absence of $1,25(OH)_2D_3$ or analogs (% of control).

RNA analysis

For Northern blot hybridization, guanidine thiocyanate (GTC) extracts were purified by cesium chloride gradient centrifugation[13] and 10 μg of total RNA was electrophoresed on a 1.1% agarose–formaldehyde gel.[14] Air-dried filters were baked for 2 h at 80°C and then hybridized[4] with a 537 bp *Sac*I–*Hin*dIII restriction fragment encoding exon III (the coding region) of the human PTHRP gene and with an 800 bp *Bam*HI restriction fragment of rat cyclophillin[15] as a control for the amount of RNA loaded. This restriction fragment was labeled with [^{32}P]dCTP (ICN Biomedicals Canada, Mississauga, Ontario, Canada) by the random primer method (Amersham Canada, Oakville, Ontario, Canada). Following a 24 h incubation at 42°C, filters were washed twice for 30 min with 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 carried out at −70°C using XAR film (Eastman Kodak, Rochester, NY) with two intensifying screens. Blot intensity was analyzed by laser densitometry (Ultrascan XL; LKB Instruments, Gaithersburg, MD).

Statistical analysis

All results are expressed as the mean ± SEM of replicate determinations and statistical comparisons based on one-way analysis of variance or by the Student's *t*-test. A probability value of $p < 0.05$ was considered to be significant.

Results

Effects of $1,25(OH)_2D_3$, MC903 and EB1089 on cell growth (Figure 2)

HPK1A cells (Figure 2A) were grown as described in Materials and methods, serum deprived for 24 h (basal, time 0) and stimulated with 5% FBS. In the absence of $1,25(OH)_2D_3$, MC903 or EB1089, the doubling time was approximately 42 h. However, in the presence of $1,25(OH)_2D_3$ or its analogs the doubling time was increased to about 72, with a

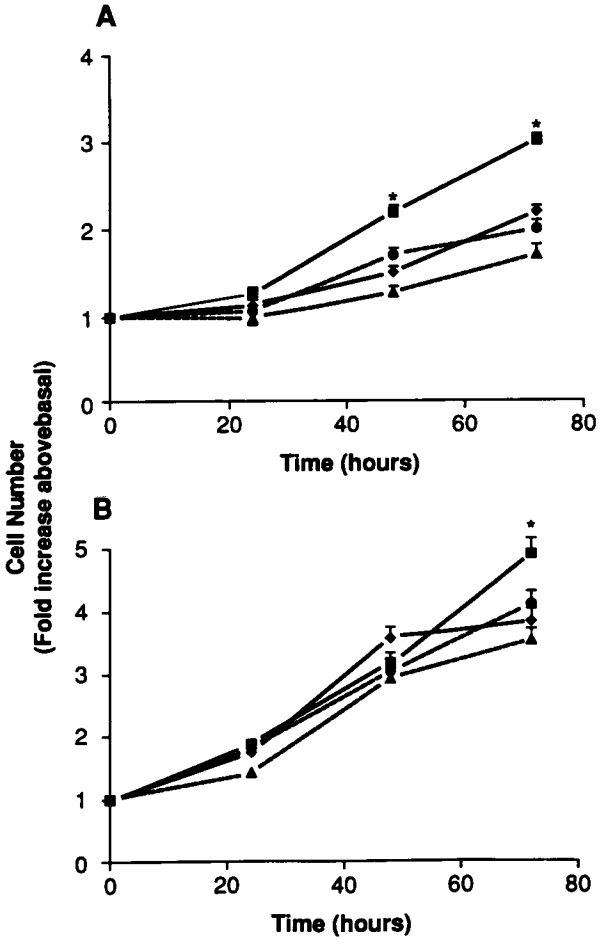

Figure 2. Effects of $1,25(OH)_2D_3$, MC903 and EB1089 on cell growth. Established HPK1A (A) and malignant HPK1A-*ras* (B) keratinocytes were grown in DMEM supplemented with 10% FBS to approximately 30% confluency (about 15–20 × 10^3 cells/cm^2). The medium was then changed to serum deprived DMEM for 24 h (quiescence) and then changed again to DMEM containing 5% FBS with 10^{-8} M $1,25(OH)_2D_3$ or MC903 and 10^{-9} M EB1089 (A) or 10^{-7} M $1,25(OH)_2D_3$ or MC903 and 10^{-8} M EB1089 (B). Cells were trypsinized at timed intervals and counted. Time zero (basal) represents the number of cells after 24 h of quiescence and was given an arbitrary value of 1. The basal cell number was 22 ± 15 × 10^3 cells/cm^3 for HPK1A and 19.8 ± 2.2 × 10^3 cells/cm^2 for HPK1A-*ras*. Cell number was then expressed as fold increase above basal and each value is the mean ± SEM of three different experiments done in sixplicate. Asterisks represent significant differences from corresponding cell numbers in the absence of $1,25(OH)_2D_3$, MC903 or EB1089. ■, Control; ◆, $1,25(OH)_2D_3$; ●, MC903; ▲, EB1089.

significant inhibition of cell growth observed at 48 and 72 h. In addition, although $1,25(OH)_2D_3$ and MC903 were about equipotent on a molar basis, only 1/10 the concentration of EB1089 (10^{-9} M) relative to $1,25(OH)_2D_3$ or MC903 (10^{-8} M) was required to produce the same effect in this system.

The doubling time of the neoplastic HPK1A-*ras* cells (Figure 2B) was much shorter (about 24 h) than that of the immortalized cells (Figure 2A). Significant growth inhibition by the vitamin D compounds was only observed at 72 h and again only 1/10 the concentration of EB1089 (10^{-8} M) was required relative to $1,25(OH)_2D_3$ or MC903 (10^{-6} M). Both $1,25(OH)_2D_3$ and EB1089 produced a dose-dependent inhibition of cell growth in both immortalized and neoplastic cell lines. However, both compounds were approximately 10–100 times more potent in inhibiting cell growth in HPK1A than in HPK1A-*ras* (Figure 3A and B).

Effects on $1,25(OH)_2D_3$ and vitamin D analogs on PTHRP secretion

The effect of $1,25(OH)_2D_3$ and its analogs on PTHRP secretion was analyzed in the same conditions as described for cell growth. Medium was collected at timed intervals and PTHRP measured using an NH_2-terminal assay. Table 2 indicates the percentage of residual PTHRP secretion from maximal stimulated levels after treatment of HPK1A or HPK1A-*ras* cells with $1,25(OH)_2D_3$ or its analogs. In immortalized HPK1A cells PTHRP inhibition was approximately 40% and was about 10–20% in HPK1A-*ras* cells. In addition, EB1089 produced a higher level of inhibition at a 10-fold lower concentration (10^{-9} M) than $1,25(OH)_2D_3$ or MC903 (10^{-8} M).

We subsequently analyzed the effects of $1,25(OH)_2D_3$ and EB1089 on PTHRP secretion over a wide range of concentrations in both cell types (Figure 4A and B). In HPK1A cells (Figure 4A) the minimal dosage producing significant inhibition of PTHRP release was 10^{-10} M for $1,25(OH)_2D_3$ and 10^{-11} M for EB1089. In contrast, the minimal dosage producing significant inhibition of PTHRP release in HPK1A-*ras* cells (Figure 4B) was 10^{-8} M for $1,25(OH)_2D_3$ and 10^{-10} M for EB1089. We also compared the effects of $1,25(OH)_2D_3$ and MC903 on PTHRP secretion in both cell systems (Figure 5A and B). Although $1,25(OH)_2D_3$ and MC903 appeared equipotent in both systems, the two compounds were at least five times more efficacious in HPK1A cells (Figure 5A) than in HPK1A-*ras* (Figure 5B).

Effect of $1,25(OH)_2D_3$ and EB1089 on PTHRP mRNA

Northern blot analysis of HPK1A-*ras* cells revealed the presence of a major transcript of 1.6 kb which

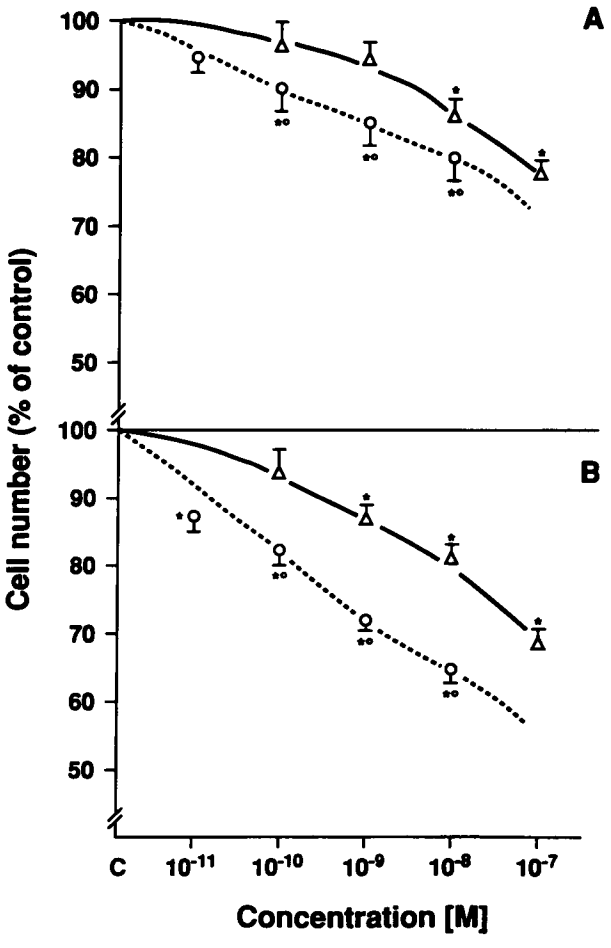

Figure 3. Dose-dependent growth inhibition of HPK1A and HPK1A-*ras* cells by $1,25(OH)_2D_3$ and EB1089. Cells were grown as described in Figure 2 and treated for 72 h with increasing concentrations of $1,25(OH)_2D_3$ (A) or EB1089 (B). Control (C) represents HPK1A or HPK1A-*ras* cells treated in DMEM with 5% FBS for 72 h. Cell number is expressed as percentage of control ± SEM of three different experiments done in sixplicate. The control cell number was 640 ± 13 × 10^3 for HPK1A and 960 ± 56 × 10^3 for HPK1A-*ras* cells. Asterisks represent significant differences from cells incubated in the absence of $1,25(OH)_2D_3$ or EB1089, and circles represent significant differences between HPK1A and HPK1A-*ras* cell number at their respective concentration. ○, HPK1A; △, HPK1A-*ras*.

was increased in the presence of 5% FBS (Figure 6). Addition of increasing concentrations of $1,25(OH)_2D_3$ or EB1089 produced a dose-dependent inhibition of PTHRP mRNA at 2 and 24 h. At 24 h, approximately 15% inhibition of PTHRP mRNA was observed with 10^{-8} M $1,25(OH)_2D_3$ and 40% inhibition of PTHRP mRNA with 10^{-8} M EB1089 which is consistent with the degree of inhibition observed with immunoassayable PTHRP protein in the conditioned medium (Figure 4B). However, as noted for inhibition of immunoassay-

Table 2. Relative effectiveness of $1,25(OH)_2D_3$ and its analogs on PTHRP inhibition in HPK1A and HPK1A-*ras* cells

	Residual PTHRP production[a]		
	$1,25(OH)_2D_3$ (10^{-8} M)	MC903 (10^{-8} M)	EB1089 (10^{-9} M)
HPK1A	65.4 ± 10.52	61.3 ± 8.95	60.3 ± 4.69
HPK1A-*ras*	89.6 ± 4.16	89.2 ± 7.52	78.1 ± 2.27

[a]Values are expressed as a % of control PTHRP concentrations obtained with 5% FBS in the absence of $1,25(OH)_2D_3$ or analogs, given an arbitrary value of 100%. The control PTHRP concentrations were 4.6 ± 0.35 and 4.92 ± 0.28 ng-eq of hPTHRP(1–34)/10^6 cells for HPK1A and HPK1A-*ras*, respectively. Concentration of EB 1089 was 10^{-9} M and of 1,25 $(OH)_2D_3$ and MC903 were 10^{-8} M.

able PTHRP, EB1089 was approximately 10 times more potent than $1,25(OH)_2D_3$ on inhibition of PTHRP gene expression. At the higher dosage of EB1089 (10^{-7} M) there was a complete inhibition of PTHRP mRNA although cell numbers were only reduced by approximately 50% of control values.

Discussion

In the present study we have first compared the effects of $1,25(OH)_2D_3$ and two of its analogs on keratinocyte cell growth. The effect of $1,25(OH)_2D_3$ on inhibition of keratinocyte cell growth has previously been demonstrated[3,16] and psoriasis, a skin disease characterized by abnormally high keratinocyte proliferation rates and poor keratinocyte differentiation, has been successfully treated both orally and topically with $1,25(OH)_2D_3$ or its derivatives.[6,17] Indeed calcipotriol (MC903) was initially developed with the aim of obtaining a more potent analog active on cell proliferation and cell differentiation but with a lower risk of inducing the classical vitamin D associated calcemic side effects. In the present study this analog was found to be equipotent to $1,25(OH)_2D_3$ on inhibition of cell growth both in immortalized (HPK1A) and neoplastic (HPK1A-*ras*) keratinocytes. The efficiency of this analog is not surprising in view of its potent antiproliferative activity both as a topical agent in psoriasis[18] and in cutaneous metastasis of breast cancers in which vitamin D receptors were expressed.[19] However, this agent has a short half-life *in vivo* and has been shown to be extensively metabolized by skin cells *in vitro*,[20] which may explain at least in part its low calcemic activity *in vivo*. For this reason, the search for low calcemic analogs with similar pharmacokinetic properties as $1,25(OH)_2D_3$ has been very active in the past few years. Some of these compounds were found to be more potent than $1,25(OH)_2D_3$ in differentiating HL60 cells[2] [$1,25(OH)_2D_3$-24 homo-D_3 and $1,25(OH)_2D_3$-22 ene-24 homo D_3] and in suppressing cell growth and inducing phagocytic activity in a mouse myelomonocytic leukemia cell line.[21] The latter known as $1,25(OH)_2D_3$-22 oxa-D_3 or 22-oxa-calcitriol (OCT) has been shown to be a potent growth inhibitor of both estrogen receptor positive and negative breast cancer cells.[22]

In this study we have used a new analog, EB1089, whose half-life is similar to $1,25(OH)_2D_3$ *in vivo*[9] but is at least 100 times less potent than $1,25(OH)_2D_3$ on calcium mobilization.[8] In this and previous studies we have shown that neoplastic keratinocytes are relatively resistant to the growth inhibitory effect of $1,25(OH)_2D_3$ requiring at least 10 times higher concentrations of $1,25(OH)_2D_3$ to produce a similar effect.[23] The mechanism of that resistance is still elusive but is likely to occur at the level of interaction between the vitamin D receptor and its target genes, since no abnormalities could be found in the ligand binding domain or the DNA binding domain of the receptor (data not shown). In the present study, we demonstrated that EB1089 was at least 10 times more potent than $1,25(OH)_2D_3$ or MC903 on growth inhibition, suggesting that this analog may be a good candidate for clinical trials in squamous cancers. These results are in agreement with the findings of other groups showing that this analog has potent antiproliferative effects on MCF-7 breast cancer cells *in vitro* and causes a reduction of tumor growth in the nitrosomethylurea-induced rat mammary tumor model.[24] We also analyzed and compared the effects of $1,25(OH)_2D_3$ and its analogs on PTHRP expression. In parallel with their effects on cell growth these analogs displayed strong inhibitory properties on gene expression and secretion of PTHRP, a factor believed to

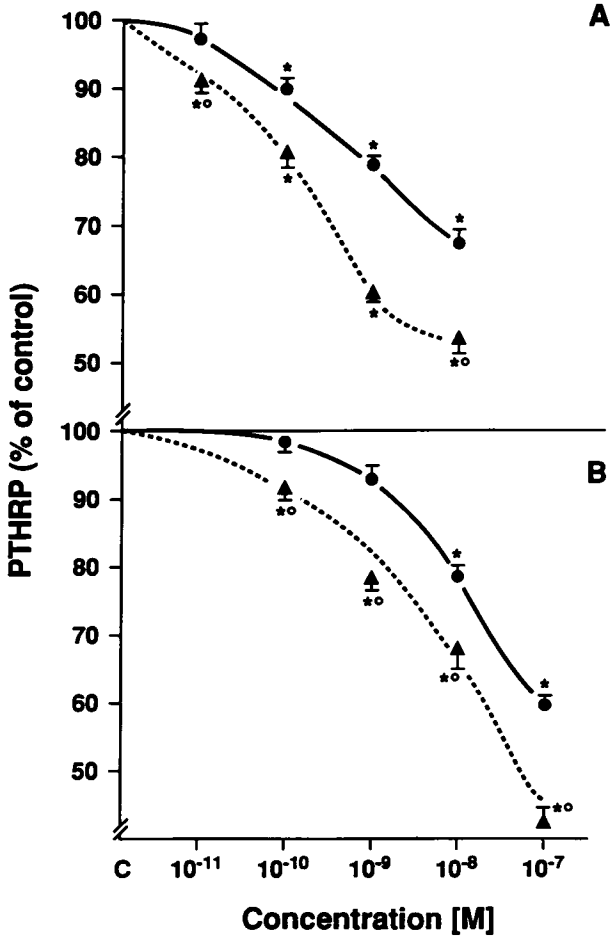

Figure 4. Effects of $1,25(OH)_2D_3$ and EB1089 on PTHRP production. HPK1A (A) and HPK1A-*ras* (B) cells were grown as described in Figure 2, and treated with increasing concentrations of $1,25(OH)_2D_3$ or EB1089. Control (C) represents PTHRP concentrations in conditioned medium from cells treated with DMEM plus 5% FBS. The conditioned medium was collected from HPK1A and HPK1A-*ras* cells at 24 h for PTHRP measurement by radioimmunoassay (RIA). The control values were 3.98 ± 0.3 and 4.26 ± 0.18 ng-eq of hPTHRP(1–34)/10^6 cells for HPK1A and HPK1A-*ras*, respectively. PTHRP levels are expressed as percentage of control ± SEM of three difference experiments done in sixplicate. All results were corrected for cell number. Asterisks represent significant differences from control, and circles represent significant differences between $1,25(OH)_2D_3$ and EB1089 at their respective concentrations. ●, $1,25(OH)_2D_3$; ▲, EB1089.

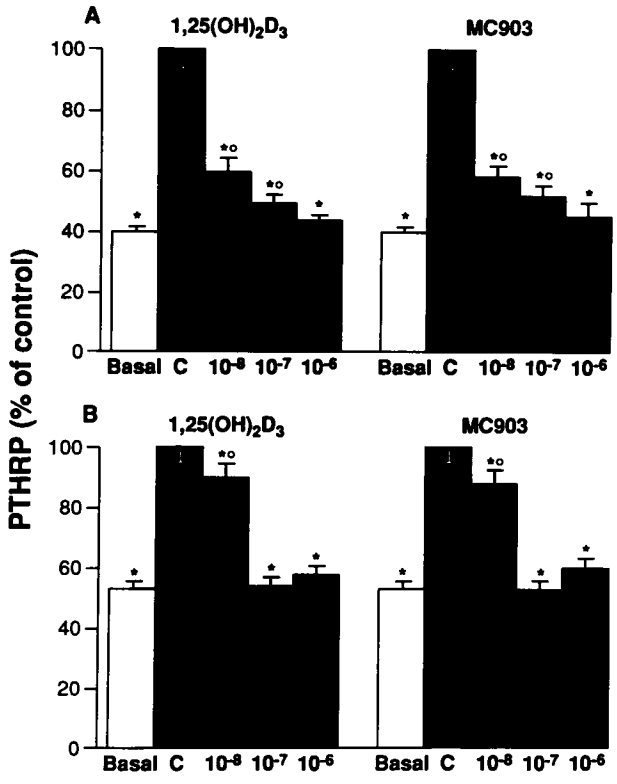

Figure 5. Effects on $1,25(OH)_2D_3$ and MC903 on PTHRP production. HPK1A (A) and HPK1A-*ras* (B) cells were grown as described in Figure 2, and treated with increasing concentrations of $1,25(OH)_2D_3$ or MC903. Basal represents PTHRP concentrations in conditioned medium from cells treated with DMEM only, and were 0.68 ± 0.03 and 1.82 ± 0.04 ng-eq of hPTHRP (1–34)/10^6 cells for HPK1A and HPK1A-*ras*, respectively. Control (C) represents PTHRP concentrations in conditioned medium from cells treated with DMEM plus 5% FBS and were 4.35 ± 0.32 and 3.82 ± 0.17 ng-eq of hPTHRP(1–34)/10^6 cells for HPK1A and HPK1A and HPK1A-*ras*, respectively. The conditioned medium was collected from HPK1A and HPK1A-*ras* cells at 24 h for PTHRP measurement by RIA. PTHRP levels are expressed as percentage of control and each value represents the mean ± SEM of three different experiments done in sixplicate. All results were corrected for cell number. Asterisks represent significant differences from control and circles represent significant differences from basal.

mediate hypercalcemia in the vast majority of epithelial cancers. However, we found that both $1,25(OH)_2D_3$ and EB1089 were slightly more potent in inhibiting PTHRP secretion than on PTHRP mRNA expression, suggesting that these compounds may have additional properties such as modulation of PTHRP production at the post-transcriptional level. In the present study, we found that EB1089 strongly inhibits PTHRP production and expression, being at least 10 times more potent than $1,25(OH)_2D_3$ after normalization for inhibition of cell growth. Since neoplastic keratinocytes are relatively resistant to $1,25(OH)_2D_3$ inhibition of PTHRP as compared with their non-transformed counterpart, this increased potency could be useful in inhibiting PTHRP expression *in vivo*. Recently, we have exploited this inhibitory effect on PTHRP

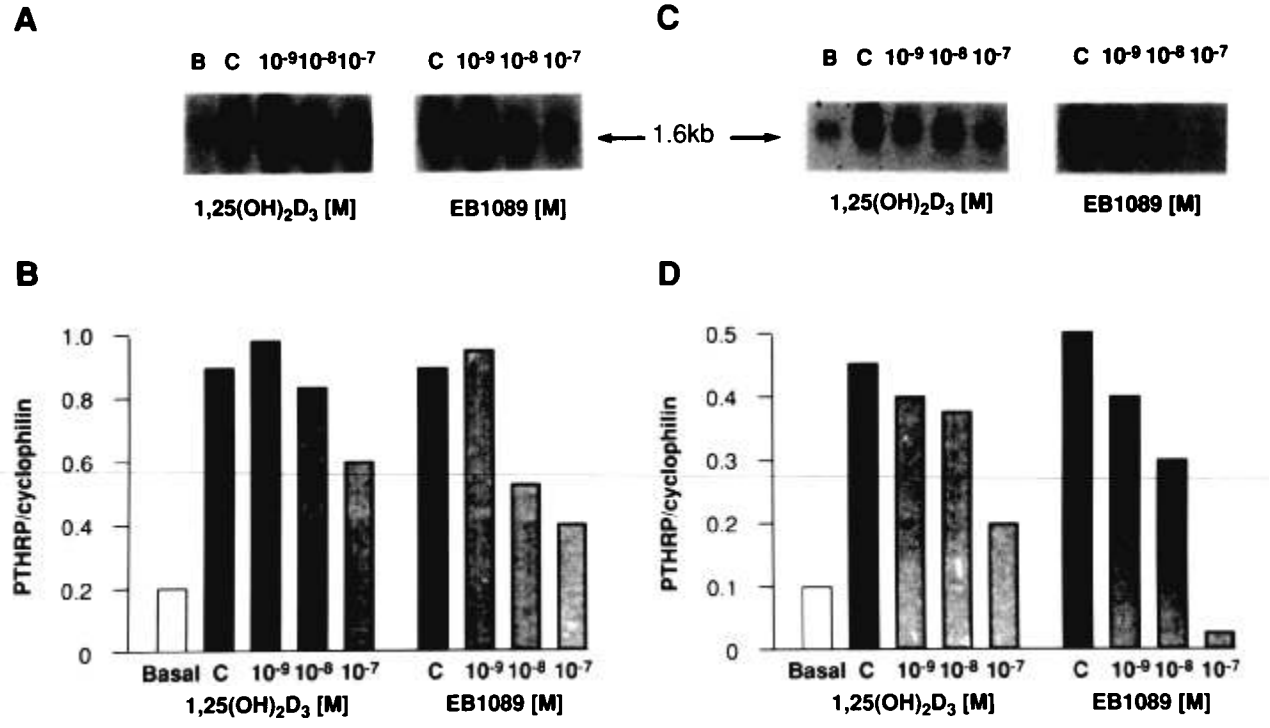

Figure 6. Northern blot analysis of PTHRP mRNA in neoplastic HPK1A-*ras* cells. Filters were hybridized as described in Materials and methods with a ^{32}P-labeled PTHRP probe and with a ^{32}P-labeled cyclophilin probe as a control for RNA loading. The lower panels represent the densitometric ratios of PTHRP/cyclophilin mRNA seen in the upper panels. The arrow represents the 1.6 kb PTHRP mRNA. Influence of increasing concentrations of $1,25(OH)_2D_3$ and EB1089 on PTHRP mRNA is shown at 2 h (panels A and B) and at 24 h (panels C and D). Basal (B) and control (C) represent the amount of PTHRP mRNA in the absence and presence of 5% FBS, respectively. Each lane contained 10 μg of total cellular RNA. These results are representative of three different experiments.

production, which is noted *in vitro* by controlling hypercalcemia in an animal model *in vivo*, the rat Leydig cell cancer model. Infusion of this analog into Fisher rats bearing the H500 Leydig cell tumor (a rat model that mimics human malignancies associated with hypercalcemia) has resulted in reduction *in vivo* of PTHRP production, normalization of serum calcium in these animals and prolongation of their survival time.[25] However, the model of human epithelial carcinogenesis described in the present study may be more relevant for predicting the *in vivo* effectiveness of these analogs in human cancers.

In conclusion, our studies strongly suggest that analogs of this class could be useful both in normalizing hypercalcemia and in controlling growth of human squamous cancer. Further studies are now underway to evaluate this hypothesis.

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References

1. Reichel H, Koeffler HP, Norman AW. The role of the vitamin D endocrine system in health and disease. *N Engl J Med* 1989; **320**: 980–91.
2. Rigby WFC, Stacy T, Fanger MW. Inhibition of T lymphocyte mitogenesis by 1,25 dihydroxyvitamin D_3 (calcitriol). *J Clin Invest* 1984; **74**: 1451–5.
3. Smith EL, Walworth NC, Holick MF. Effect of 1 alpha,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol* 1986; **86**: 709–14.
4. Kremer R, Karaplis AC, Henderson J, *et al.* Regulation of parathyroid hormone-like peptide in cultured normal human keratinocytes: effect of growth factors and 1,25 dihydroxyvitamin D_3 on gene expression and secretion. *J Clin Invest* 1991; **87**: 884–93.
5. Kremer R, Champigny C, Meerovitch K, *et al.* Negative and positive regulatory regions in the rat parathyroid hormone regulated peptide (PTHRP) gene promoter. *J Bone Min Res* 1993; **8**: A67, S133.

6. Binderup L, Bramm E. Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation *in vitro* and on calcium metabolism *in vivo*. *Biochem Pharmacol* 1988; **37**: 889–95.
7. Gumowski-Sunek D, Rizzoli R, Saurat JH. Treatment of plaque-type psoriasis by calcipotriol (MC903): therapeutic efficacy and systemic effect on calcium metabolism. *Dermatologica* 1990; **181**: 176–80.
8. Binderup E, Calverley MJ, Binderup L. Synthesis and biological activity of 1 alpha-hydroxylated vitamin D analogues with polyunsaturated side chains. In: Norman AW, Bouillon R, Thomasset M, eds. *Vitamin D: gene regulation, structure–function analysis and clinical application: Proc. 8th Workshop on Vitamin D.* Berlin: de Gruyter 1991: 192–3.
9. Mathiasen IS, Colston KW, Binderup L. EB1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. *J Steroid Biochem Mol Biol* 1993; **46**: 365–71.
10. Dürst M, Dzarlieva-Petrusevska RT, Boukamp P, *et al.* Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* 1987; **1**: 251–6.
11. Henderson JE, Sebag M, Rhim JS, *et al.* Dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression. *Cancer Res* 1991; **51**: 6521–8.
12. Dürst M, Gallahan D, Jay G, *et al.* Glucocorticoid-enhanced neoplastic transformation of human keratinocytes by human papillomavirus type 16 and an activated *ras* oncogene. *Virology* 1989; **173**: 767–71.
13. Chirgwin JM, Przybyla AE, Macdonald RJ, *et al.* Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979; **18**: 5294–9.
14. Kremer R, Bolivar I, Goltzman D, *et al.* Influence of calcium and 1,25-dihydroxycholecalciferol on proliferation and proto-oncogene expression in primary cultures of bovine parathyroid cells. *Endocrinology* 1989; **125**: 935–41.
15. Danielson PE, Forrs-Petter S, Brow MA, *et al.* p1β15: a cDNA clone of the rat mRNA encoding cyclophillin. *DNA* 1988; **7**: 261–7.
16. Hosomi J, Hosoi J, Abe E, *et al.* Regulation of terminal differentiation of cultured mouse epidermal cells by 1,alpha,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown in serum-free conditions. *J Invest Dermatol* 1983; **86**: 709–14.
17. Holick MF. Will 1,25 dihydroxyvitamin D_3, MC903 and their analogues herald a new pharmacologic era for the treatment of psoriasis. *Arch Dermatol* **125**: 1692–7.
18. Kragballe K. Treatment of psoriasis by the topical application of the novel cholecalciferol analogue calcipotriol (MC903). *Arch Dermatol* 1989; **125**: 1647–52.
19. Bower M. Topical application of calcipotriol (MC903) inhibits cutaneous metastasis of breast cancer. In: Norman AW, Bouillon R, Thomasset M, eds. *Vitamin D: gene regulation, structure–function analysis and clinical application: Proc 8th Workshop on Vitamin D.* Berlin: de Gruyter 1991; 247.
20. Masuda S, Strugnell S, Calverley MJ, *et al. In vitro* metabolism of the antipsoriatic vitamin D analog, calcipotriol, in two cultured human keratinocyte models. *J Biol Chem* 1994; **269**: 4794–803.
21. Ostrem VK, Tanaka Y, Prahl J, *et al.* 24 and 26-homo-1,25 dihydroxyvitamin D_3: Preferential activity in inducing differentiation of human leukemia cells HL-60 *in vitro*. *Proc Natl Acad Sci USA* 1987; **84**: 89–95.
22. Nakano T, Nishii Y, Matsumoto T, *et al.* 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* 1991; **129**: 832–7.
23. Sebag M, Henderson JE, Rhim JS, *et al.* Relative resistance to 1,25-dihydroxyvitamin D_3 in a keratinocyte model of tumor progression. *J Biol Chem* 1992; **267**: 12162–7.
24. Colston KW, Mackay AG, Chander S, *et al.* Novel vitamin D analogues suppress tumor growth *in vivo*. In: Norman AW, Bovillon R, Thomasset M, eds. *Vitamin D: gene regulation, structure–function analysis and clinical application: Proc 8th Workshop on Vitamin D.* Berlin: de Gruyter 1991: 465–6.
25. Haq M, Kremer R, Goltzman D, *et al.* A vitamin D analogue (EB1089) inhibits parathyroid hormone related peptide production and prevents the development of malignancy-associated hypercalcemia *in vivo*. *J Clin Invest* 1993; **91**: 2416–22.

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