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EB1089: A NEW VITAMIN D ANALOGUE THAT INHIBITS THE GROWTH OF BREAST CANCER CELLS *IN VIVO* AND *IN VITRO*

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Abstract—EB1089 is a novel vitamin D analogue which has been tested for its effects on breast cancer cell growth *in vitro*, using the established human breast cancer cell line MCF-7, and *in vivo* on the growth of established rat mammary tumours. Both EB1089 and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) inhibited MCF-7 cell proliferation with the synthetic analogue being at least an order of magnitude more potent than the native hormone. *In vivo* anti-tumour effects were investigated using the *N*-methyl-nitrosourea-induced rat mammary tumour model. Oral treatment with EB1089 was tested at three doses. With the lower dose, significant inhibition of tumour growth was seen in the absence of a rise in serum calcium. The same dose of 1,25-$(OH)_2D_3$ had no effect on tumour growth but caused hypercalcaemia. With the higher dose of EB1089, striking tumour regression was seen although serum calcium rose. This report demonstrates that EB1089 possesses enhanced anti-tumour activity coupled with reduced calcaemic effects relative to 1,25-$(OH)_2D_3$ and thus may have therapeutic potential as an anti-tumour agent.

In recent years an increasing body of evidence has suggested that the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (calcitriol, 1,25-$(OH)_2D_3$‖), exerts effects on a variety of tissues apparently unrelated to calcium homeostasis [1]. This steroid hormone has been shown to promote cellular differentiation and inhibit proliferation of haematopoietic cells [2–4], cancer cells [5–7] and the epidermis [8]. In addition, effects on cellular oncogene transcription and growth factor receptor expression have been demonstrated *in vitro* [9, 10]. Previous studies have shown that this stimulation of differentiated cell function and inhibition of cellular proliferation is mediated via the intracellular receptor for 1,25-$(OH)_2D_3$ [11, 12].

Studies *in vivo* have shown that animal xenografts of lung and colon tumours and melanomas regress when animals are treated with large doses of 1,25-$(OH)_2D_3$ [13] although animals were maintained on a low calcium diet to reduce toxicity due to hypercalcaemia. Calcitriol (1,25-$(OH)_2D_3$) and its synthetic analogue 1α-hydroxyvitamin D_3 [1α(OH)D_3, which is converted to 1,25-$(OH)_2D_3$ *in vivo*] prolonged the survival time of mice inoculated with murine leukaemia cells [14] and 1α(OH)D_3 also inhibited the progression of carcinogen-induced rat mammary tumours [15].

Such studies have suggested the possible clinical use of 1,25-$(OH)_2D_3$ in the treatment of hyperproliferative disorders such as leukaemia and psoriasis. However, a major drawback to considering conventional vitamin D metabolites as therapeutic agents is that they produce hypercalcaemia at doses of more than a few micrograms per day. Recently, a number of laboratories have developed synthetic vitamin D analogues which are active in promoting cellular differentiation and inhibiting cell growth but with reduced calcaemic activity [16–18]. One compound, calcipotriol, has been shown to be effective in topical treatment of psoriasis [19] and we have shown that 25% of patients with locally advanced or cutaneous metastatic breast cancer treated topically with calcipotriol showed a response of the treated lesion [20]. Using a rat mammary tumour model, we have shown that intraperitoneal treatment of tumour-bearing animals with calcipotriol produced only modest inhibition of tumour growth [21]. However, the metabolic half-life of calcipotriol in the rat is in the order of minutes [22]. We have therefore studied *in vivo* anti-tumour effects of new vitamin D analogues which are not degraded as rapidly. We report here studies with a new vitamin D analogue EB1089 which, like calcipotriol, is characterized by a modification in the C17 side chain of the vitamin D molecule.

MATERIALS AND METHODS

Compounds. Crystalline 1,25-$(OH)_2D_3$ was a generous gift from Dr W. Meier, Hoffmann-La Roche and Co. (Basel, Switzerland). EB1089 was synthesized in the Department of Chemical Research

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‖ Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxycholecalciferol; NMU, *N*-methyl-nitrosourea; VDR, vitamin D receptor.

at Leo Pharmaceutical Products as described previously [23]. For the *in vitro* studies compounds were dissolved in absolute ethanol. For the *in vivo* studies compounds were dissolved in propylene glycol. 1,25-Dihydroxy-[26,27-*methyl*-^{3}H]cholecalciferol (180 Ci/mmol), 25-hydroxy-[23,24-*N*-^{3}H]-cholecalciferol (180 Ci/mmol) and [^{3}H]thymidine (5 Ci/mmol) were obtained from Amersham International (Amersham, U.K.). Other radioinert chemicals were purchased from the Sigma Chemical Co. (Poole, U.K.). Tissue culture medium and reagents were obtained from Gibco (Paisley, U.K.). Tamoxifen was obtained from ICI (Macclesfield, U.K.).

Cellular effects in vitro. MCF-7 human breast cancer cells were grown in Dulbecco's modification of Eagles minimal essential medium (DMEM) supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% foetal calf serum.

To examine the binding of the vitamin D derivatives to the intracellular receptor for 1,25-$(OH)_2D_3$ (VDR), cells from confluent monolayers were sonicated in hypertonic buffer; 300 mM KCl, 10 mM Tris–HCl, 1 mM EDTA, 10 mM sodium molybdate, 4 mM dithiothreitol, pH 7.4 (KTEDM) plus 500 Trypsin Inhibitor Units (TIU)/mL Trasylol solution. Aliquots (200 μL) of KTEDM extracts were incubated with approximately 10,000 dpm [^{3}H]1,25-$(OH)_2D_3$ and increasing concentrations of radioinert 1,25-$(OH)_2D_3$ or EB1089. After incubating at 4° for 18 hr the bound and free sterols were separated by the hydroxylapatite method as described previously [24]. Binding of vitamin D derivatives to serum binding proteins was assessed as described previously [21].

For studies on cell proliferation, suspensions of MCF-7 cells were adjusted to 1×10^4 cells/mL in RPMI 1640 medium containing 2.5% charcoal stripped foetal calf serum, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and also 1×10^{-9} M triiodothyronine, 10 ng/mL hydrocortisone, 5 μg/mL transferrin and 6 μg/mL bovine insulin. The cells were plated in 24-well plates (Falcon) and incubated for 24 hr at 37° in a humidified 5% CO_2 atmosphere. Medium was removed and fresh medium containing 1,25-$(OH)_2D_3$ or EB1089 (10^{-8} to 10^{-11} M) was added. Control cultures containing ethanol vehicle (0.1%) were incubated in parallel. Cells were cultured for up to 10 days and medium was changed on alternate days. Cell cultures were assayed for [^{3}H]thymidine incorporation at various times after addition of vitamin D derivatives by addition of 0.5 μCi/mL [^{3}H]thymidine to the incubation medium for the last 4 hr of culture. After labelling, the medium was aspirated and cell layers were washed three times with ice-cold phosphate-buffered saline containing 1 mM radioinert thymidine. The amount of radioactivity incorporated into trichloroacetic acid precipitable material was determined as described previously [25] with six replicate cultures for each concentration tested.

Treatment of tumour bearing rats. An inbred strain of virgin female Ludwig/Wister/Olac rats bearing mammary tumours induced by nitrosomethylurea (NMU; Harlan OLAC Ltd, Oxon, U.K.) were kept as described previously [26]. Rats bearing at least one assessable tumour (>10 mm in diameter) were randomly assigned to treated or control groups. Vitamin D derivatives were given in propylene glycol and control rats received vehicle alone. Rats were treated daily by gavage for 28 days and tumour volume was assessed weekly. Tumour volume was determined by measuring the two largest diameters at right angles, using vernier calipers. From these values total tumour volume was calculated using the formula $1/6\pi\{(D_1 \times D_2)^{3/2}\}$ (where D_1 and D_2 are the two diameters). The percentage change in total tumour volume compared with tumour volume at start of treatment was calculated for each rat. Animals whose tumours showed signs of ulceration or in which tumour burden became excessive (>10% body weight) were culled. At the end of each experiment, animals were exsanguinated under halothane anaesthesia. Tumours were excised, immediately frozen in liquid nitrogen and stored at −70°. Serum was stored at −20° until analysed.

Other methods. Serum calcium and albumin were measured on a Technicon RA-1000 analyser.

Statistical methods. Percentage change in total tumour volume at each week of each study was compared between groups using the non-parametric Mann–Whitney U test. Comparisons of the biochemical parameters and *in vitro* studies used the unpaired Student's *t*-test.

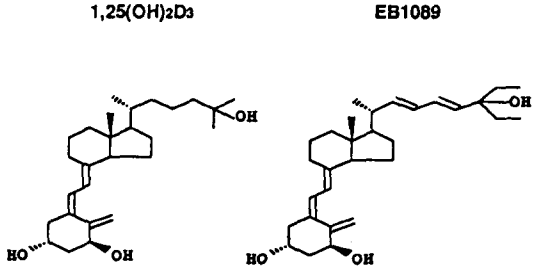

Fig. 1. Structural formulae of 1,25$(OH)_2D_3$ and its new synthetic analogue EB1089.

RESULTS

*EB*1089

EB1089 is 1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′-ethyl-5′-hydroxy-hepta-1′(*E*),3′(*E*)-dien-1′-yl)-9,10-secopregna-5(*Z*),7(*E*),10(19)-triene. Its structure, and that of 1,25-dihydroxyvitamin D_3 are shown in Fig. 1.

*Binding of EB*1089 *to the vitamin D receptor*

The ability of EB1089 to bind to VDR was determined by the displacement of [^{3}H]1,25-$(OH)_2D_3$ from KTEDM extracts of MCF-7 cells. Figure 2 shows that half-maximal displacement of [^{3}H]1,25-$(OH)_2D_3$ was obtained with EB1089 at a concentration of 6×10^{-10} M and with 1,25-$(OH)_2D_3$ at a concentration of 1.5×10^{-10} M. Thus, EB1089 interacts with VDR in a manner similar to 1,25-$(OH)_2D_3$, although the affinity of the receptor for

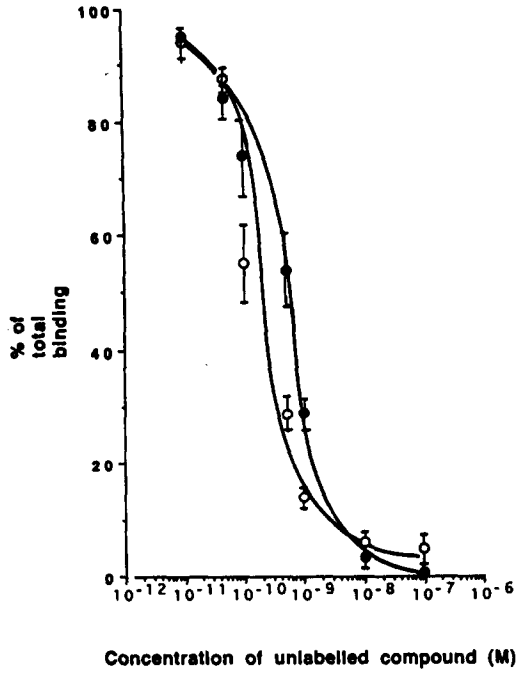

Fig. 2. Vitamin D receptor binding of $1,25(OH)_2D_3$ and EB1089. KTEDM extracts of MCF-7 cells were incubated with 10,000 dpm $[^3H]1,25(OH)_2D_3$ in the presence or absence of increasing amounts of radioinert $1,25(OH)_2D_3$ (○) and EB1089 (●). Results are expressed as % (±SEM) of radioactivity bound in the absence of added compounds and are representative of three separate experiments.

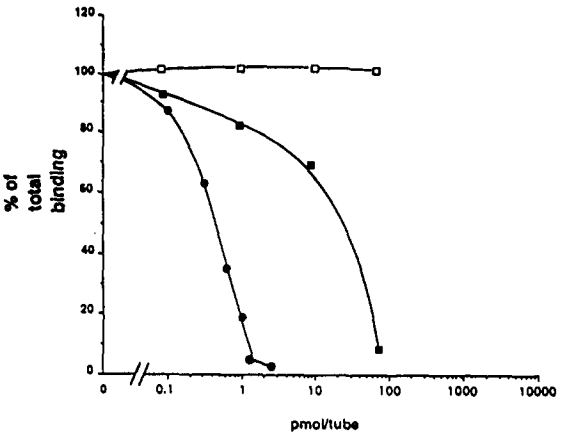

Fig. 3. Binding of vitamin D derivatives to serum binding proteins. The displacement of $[^3H]25(OH)D_3$ from rat serum by increasing concentrations of radioinert $25(OH)D_3$ (●), $1,25(OH)_2D_3$ (■) and EB1089 (□) is expressed as % of radioactivity bound in the absence of added compounds.

the synthetic analogue appears to be somewhat reduced relative to the native hormone.

To compare the abilities of EB 1089 and $1,25-(OH)_2D_3$ to bind to transport proteins in rat serum, competition studies were carried out. Figure 3 shows that little displacement of $[^3H]25(OH)D_3$ from serum binding proteins was obtained with EB1089 over the concentration range tested while $1,25-(OH)_2D_3$ was a less potent competitor for $[^3H]25(OH)D_3$ binding than $25(OH)D_3$ itself.

Effects of EB1089 on breast cancer cell proliferation in vitro

In order to compare effects of EB1089 and $1,25-(OH)_2D_3$ on MCF-7 breast cancer cell growth, cells were grown for 4–10 days in the presence or absence of the vitamin D derivatives and assessed for $[^3H]$-thymidine incorporation (Fig. 4). Significant dose-dependent inhibition of cell growth was seen with both compounds. EB1089 at a final concentration of 5×10^{-10} M produced greater than 50% inhibition of $[^3H]$thymidine incorporation after 4, 7 and 10 days in culture (Fig. 4A–C) and significant inhibition of growth was seen at 5×10^{-11} M at all three time points. In contrast, $1,25-(OH)_2D_3$ produced a 50% inhibition of $[^3H]$thymidine incorporation only at 5×10^{-9} M and significant inhibition of cell growth could only be shown with 5×10^{-10} M at all three time points. Effects on cell proliferation were also examined by assessing numbers of viable cells in treated and control cultures by trypan blue dye exclusion (Fig. 4D). The findings confirm that EB1089 is a more potent inhibitor of breast cancer cell proliferation than the native hormone by at least an order of magnitude.

Effects of EB1089 on rat mammary tumour progression

The NMU-induced rat mammary tumour model was used to study effects of EB1089 and $1,25-(OH)_2D_3$ on tumour growth *in vivo*. Rats were treated orally with EB1089 at three doses (0.5, 1.0 or 2.5 μg/kg body weight) or with $1,25-(OH)_2D_3$ (0.5 μg/kg body weight). Because of difficulties in treating large numbers of animals concurrently, these dose regimens were tested in three separate trials and results compared to respective control groups. As shown in Table 1, EB1089 caused a dose-dependent inhibition of tumour progression. Results with the lower dose of EB1089 are shown in Fig. 5. Significant inhibition of tumour progression was observed after 28 days of treatment ($P = 0.006$, Mann–Whitney U test) in the absence of any significant rise in serum calcium concentration (Table 1). At 28 days of treatment, mean tumour volume in the group receiving the lower dose of EB1089 was approximately half that in the control group (data not shown). With the same dose of $1,25-(OH)_2D_3$ no significant effect on tumour growth was observed (Table 1). At 28 days of treatment, the percentage change in tumour volume from that recorded at the start of treatment was not significantly different from that seen in the control group ($P = 0.142$, Mann–Whitney U test). However, this treatment produced marked hypercalcaemia with a mean serum calcium concentration of 3.0 ± 0.05 mmol/L (Table 1). With the higher dose of EB1089 (2.5 μg/kg), dramatic

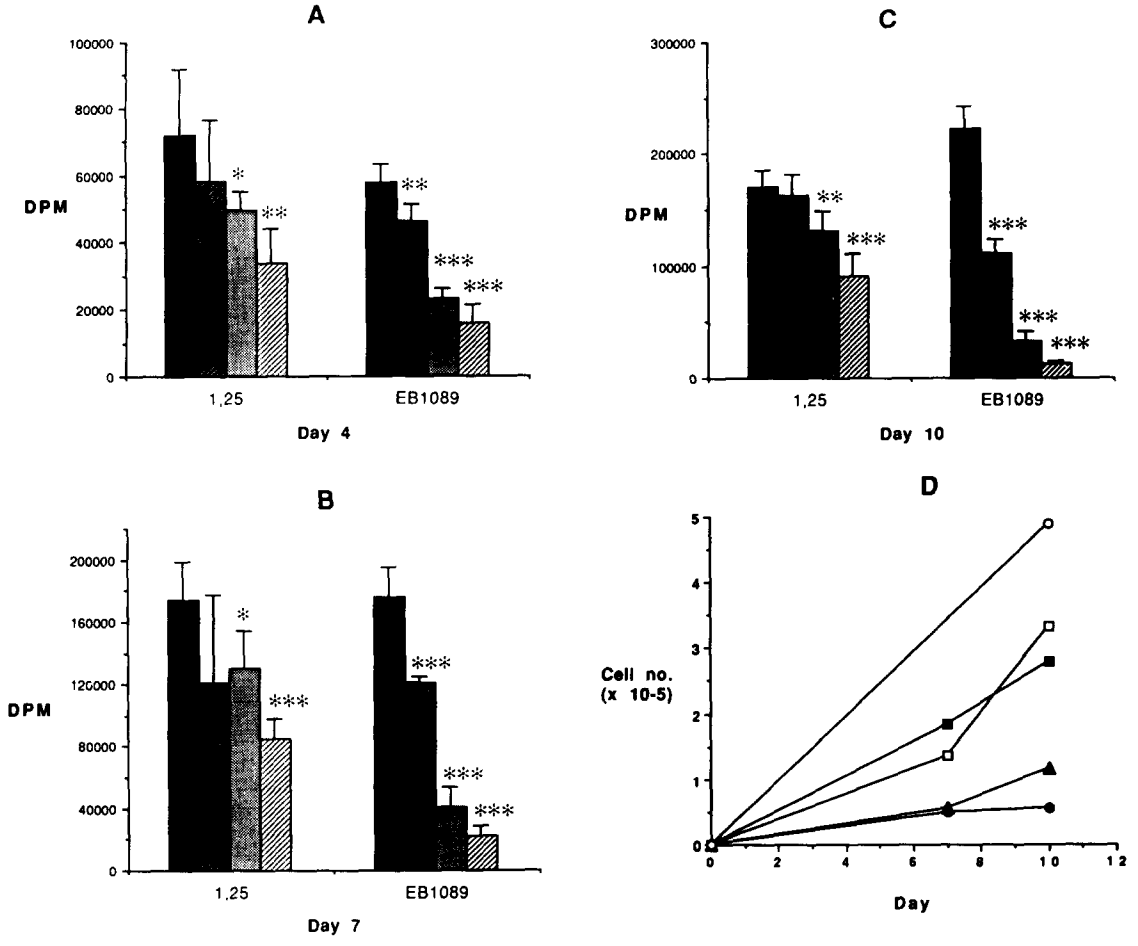

Fig. 4. Antiproliferative effects of $1,25(OH)_2D_3$ and EB1089 on MCF-7 breast cancer cells. Proliferation was assessed by [^{3}H]thymidine incorporation after 4 (A), 7 (B), and 10 (C) days in culture, in the presence or absence of increasing amounts of $1,25\text{-}(OH)_2D_3$ or EB1089 as described in Materials and Methods. (■) Control, (▨) 5×10^{-11} M, (▩) 5×10^{-10} M, (▨) 5×10^{-9} M. Results are the mean ± SD of 6 replicates and are representative of three separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. Proliferation was also assessed by trypan blue dye exclusion as described in Materials and Methods (D). Control (○), 5×10^{-10} M $1,25\text{-}(OH)_2D_3$ (□), 5×10^{-9} M EB1089 (●), 5×10^{-10} M EB1089 (▲), 5×10^{-11} M EB1089 (■). Results are means of four replicate estimations.

tumour regression was seen (Fig. 6); 80% of the treated rats had tumours which regressed by more than half at the end of the treatment period, although mean serum calcium concentration was significantly increased in treated animals (Table 1). The effects of tamoxifen on tumour growth are also shown in Table 1. Treatment of tumour-bearing rats with the anti-oestrogen at a dose of 1.0 mg/kg body weight/day produced significant tumour regression as expected and response was similar to the 1 μg/kg dose of EB1089 tested. Animals treated with the two higher doses of EB1089 displayed weight loss (mean 14% and 19%, respectively). Tumour regression was not related to weight loss as animals treated with $1,25\text{-}(OH)_2D_3$ also lost weight in the absence of significant inhibition of tumour regression. No marked weight loss was observed with the lower dose of EB1089 although this treatment regimen caused significant inhibition of tumour growth.

DISCUSSION

The novel synthetic vitamin D analogue EB1089 is a potent inhibitor of the proliferation of breast cancer cells *in vitro* and *in vivo* with a reduced calcaemic activity relative to $1,25\text{-}(OH)_2D_3$, the native hormone. This compound produced a marked inhibition of proliferation in MCF-7 breast cancer cells in culture. Effects were dose dependent and the degree of inhibition was greater than that seen with $1,25\text{-}(OH)_2D_3$ by at least an order of magnitude.

More importantly, EB1089 strikingly inhibited the growth of NMU-induced rat mammary tumours *in vivo* in a dose-dependent manner. At a dose of 0.5 μg/kg body weight, EB1089 inhibited tumour growth in the absence of a rise in serum calcium, whereas this dose of $1,25\text{-}(OH)_2D_3$ did not inhibit the growth of these tumours and produced marked hypercalcaemia. These results demonstrate that

Table 1. Effect of oral administration of vitamin D analogues on NMU-induced tumours

Treatment	No. of rats	Mean % change in tumour volume from day 0* (± SEM)	% Change from day 0 comparison	Serum calcium (mmol/L) (mean ± SEM)	Serum albumin (g/L) (mean ± SEM)	% Change in body weight from day 0¶
EB1089 (0.5 μg/kg)	15	−7(±16)†	P = 0.006	2.68 ± 0.1	38.3 ± 1.5§	−3 ± 2.1
EB1089 (1.0 μg/kg)	12	−26(±26)	P = 0.004	3.06 ± 0.07‖	39.4 ± 1.0‖	−14 ± 2.2‖
EB1089 (2.5 μg/kg)	14	−74(±10)	P = 0.0001	3.51 ± 0.07‖	32.9 ± 0.6‖	−19 ± 2.2‖
Tamoxifen (1 mg/kg)	11	−33(±38)	P = 0.007	2.46 ± 0.09	38.9 ± 0/8‖	−8 ± 1.2
$1,25(OH)_2D_3$ (0.5 μg/kg)	15	+80(±67)‡	P = 0.142	3.0 ± 0.05‖	38.9 ± 0.9‖	−10 ± 2.0‖

Tumour bearing rats were treated with EB1089, tamoxifen or propylene glycol vehicle daily p.o. at the doses shown for 28 days.

* Mean % change in tumour volume in control groups ranged from +80 to +201.

† Negative values indicate tumour regression.

‡ Positive value indicates tumour progression.

§ $P < 0.01$, ‖ $P < 0.005$: significance of differences from respective control groups.

¶ Negative values indicate weight loss.

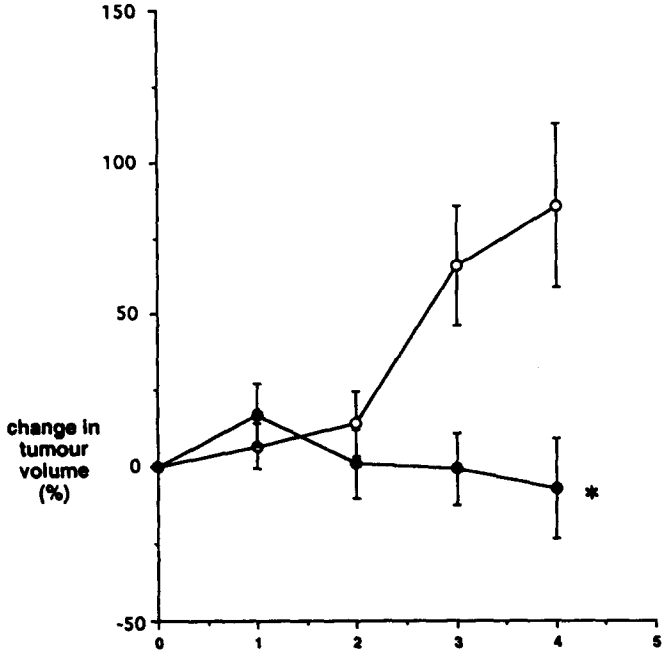

Fig. 5. Figure 5 shows the effect of daily treatment with EB1089 (●) at a dose of 0.5 μg/kg body weight and control (○) on the growth of NMU-induced rat mammary tumours. Results are expressed as the percentage change in tumour volume from day 0 and are shown as means ± SE. Statistical comparisons were made using the non-parametric Mann–Whitney U test, *P = 0.006. Serum calcium and albumin concentrations at 28 days are given in Table 1.

EB1089 possesses more potent anti-tumour effects and decreased calcium mobilizing effects relative to $1,25\text{-}(OH)_2D_3$. A higher dose of EB1089 had more profound inhibitory effects on tumour growth—more than 80% of treated rats had tumours which regressed by more than 50%, a response rate similar to that seen with tamoxifen in the present study and reported elsewhere [26]. However, serum calcium

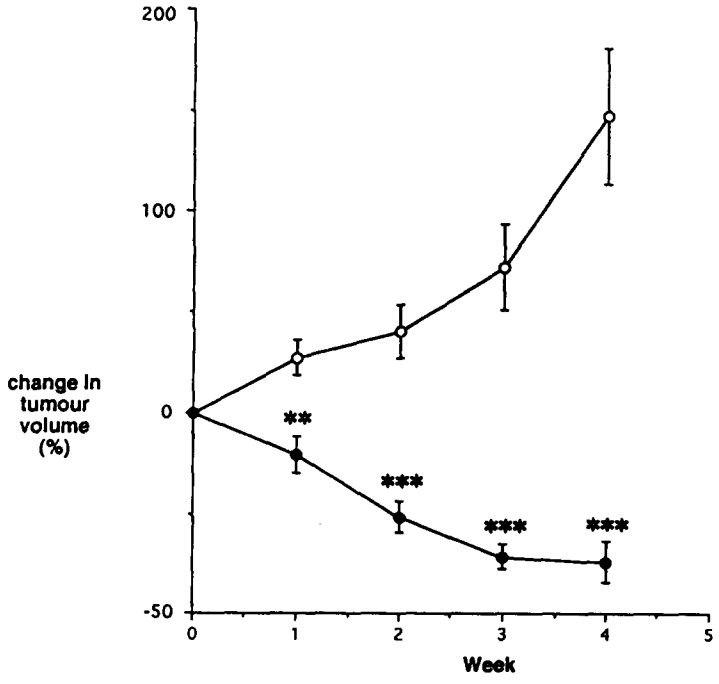

Fig. 6. Figure 6 shows the effect of treatment with EB1089 at the higher dose of 2.5 μg/kg body weight (●) and control (○) on the growth of NMU-induced rat mammary tumours. Results are again expressed as the percentage change in tumour volume from day 0 and are shown as means ± SE. Statistical comparisons were made using the non-parametric Mann–Whitney U test, ***$P < 0.005$. Serum calcium and albumin concentrations at 28 days are given in Table 1.

was significantly increased in animals treated with 2.5 μg/kg EB1089.

The present study shows that a synthetic vitamin D analogue may have enhanced anti-tumour activity and reduced calcaemic activity *in vivo* in an animal model of breast cancer. Other studies of vitamin D analogues have evaluated anti-proliferative versus calcaemic activity with *in vitro* methods. The human leukaemia cell line HL-60 has been extensively utilized to assess effects of conventional vitamin D metabolites and synthetic analogues on cell growth and differentiation [27], while actions of these compounds on calcium homeostasis *in vivo* have generally been studied in chickens [28] or rodents [18, 29]. Studies have focused on the profile of activities of synthetic compounds which are characterized by a modification of the vitamin D molecule at the C17 side chain. Introduction of fluoro groups has been reported to enhance modestly the ability of vitamin D analogues to induce differentiation [30]. The compound 1,25-$(OH)_2$-22-oxa vitamin D_3, in which an oxygen atom is substituted for the methylene group at C22, has been reported to be approximately equipotent with 1,25-$(OH)_2D_3$ in promoting cell differentiation coupled with reduced effects on calcium mobilizing ability [17]. Recently it has been reported that this analogue retards the growth of breast cancer xenografts developed in athymic mice [31], although tumour regression was not seen and no direct comparison with effects of 1,25-$(OH)_2D_3$ was made as in the present study. A series of 26- and 24-homologated 1,25-dihydroxyvitamin D_3 compounds and their delta-22-derivatives have been synthesized and appear to have enhanced activity in HL60 cells [32]. Norman *et al.* [33] reported a comparison of effects of eight synthetic analogues on HL60 cells. One analogue, 1,25-$(OH)_2$-16-ene 23-yne vitamin D_3 and its hexadeutero form was reported to be several fold more potent than the parent compound.

Few data are available regarding effects of synthetic analogues on the growth and differentiation of epithelial cells. Calcipotriol (MC903) contains a cyclopropyl substitution in the side chain and appears to be equipotent with 1,25-$(OH)_2D_3$ in promoting the differentiation of U937 leukaemia cells [18] and normal keratinocytes [34] and also in inhibiting the growth of MCF-7 breast cancer cells [21]. In clinical trials this compound has been shown to be effective in the treatment of psoriasis vulgaris [19], a disorder involving abnormal proliferation and differentiation of epidermal cells. It has been reported that 80–90% of primary breast tumour biopsy specimens contain VDR [35–37] and thus a substantial proportion of breast cancer patients are likely to be potentially responsive to the anti-tumour effects of vitamin D analogues. In a small series of patients with advanced breast cancer, topical calcipotriol treatment of cutaneous metastases produced a response in 25% of cases and response correlated with tumour VDR status [20]. Recently published data [38] have shown that 20-epi-vitamin D_3 analogues such as KH1060

are also potent inhibitors of human breast cancer cells *in vitro*. The strong immunosuppressive activity that characterizes these analogues with altered stereochemistry at C20 may, however, limit their usefulness as anti-tumour agents *in vivo*. These analogues are currently under evaluation in our laboratory in the NMU-induced rat mammary tumour model.

Previous studies on cell proliferation *in vitro* do not take account of possible differences in pharmacokinetics and delivery of these compounds to target tissues *in vivo*. Thus, it is now known that the metabolic half-life of calcipotriol is in the order of minutes which may largely account for its reduced calcaemic activity *in vivo* [22]. Preliminary data indicate that, in the rat, EB1089 has a half-life approximately equal to 1,25-$(OH)_2D_3$ [39], although it binds poorly to transport proteins in rat serum (Fig. 3). Intestinal absorption of both EB1089 and $1,25(OH)_2D_3$ is in the order of 50% since a given dose of each compound administered intraperitoneally causes approximately twice the increase in urinary calcium excretion that is seen after oral administration.* It appears that EB1089 binds less well to VDR in MCF-7 cells than does 1,25-$(OH)_2D_3$ while being at least an order of magnitude more potent in inhibiting cell proliferation. In contrast, *in vivo* EB1089 is less potent than 1,25-$(OH)_2D_3$ in raising serum calcium concentration but has profound anti-tumour effects whereas an equivalent dose of 1,25-$(OH)_2D_3$ is ineffective in retarding tumour growth.

How this structural modification of the vitamin D molecule leads to an altered profile of activities remains to be established. There is little evidence to suggest the existence of multiple forms of the vitamin D receptor. Current data indicate that there is a single gene for VDR [40] and a single translated receptor molecule, although differences in the phosphorylation state of the receptor have been detected by western analysis [41, 42] and some tissues appear to show mRNA size heterogeneity [43]. The possibility exists that interaction of synthetic vitamin D analogues with VDR results in alternative conformational changes in the receptor–ligand complex leading to altered transcription in a different spectrum of target genes.

In the present study we have shown that a synthetic analogue of 1,25-$(OH)_2D_3$, EB1089, possesses striking anti-tumour activity *in vivo* and *in vitro*. This compound is a potent inhibitor of breast cancer cell growth *in vitro* and produces significant inhibition of rat mammary tumour progression *in vivo* at a dose which does not raise serum calcium concentration. Higher doses of the vitamin D analogue produced striking regression of the experimental tumours which was comparable to effects seen with tamoxifen. EB1089 thus displays enhanced anti-tumour activity coupled with reduced calcaemic effects relative to conventional vitamin D derivatives. This compound opens the way for new therapeutic approaches to the treatment of breast cancer and other hyperproliferative disorders.

* I. S. Mathiasen, K. W. Colston and L. Binderup, manuscript submitted for publication.

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REFERENCES

1. Reichel H, Koeffler HP and Norman AW, The role of the vitamin D endocrine system in health and disease. *N Engl J Med* **320**: 980–991, 1989.
2. Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, Yoshiki S and Suda T, Differentiation of mouse myeloid leukemia cells induced by 1,25-dihydroxyvitamin D_3. *Proc Natl Acad Sci USA* **76**: 4990–4994, 1981.
3. Bar-Shavitz D, Teitlebaum SL, Reitsma P, Hall A, Pegg LE, Trial J and Kahn AJ, Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc Natl Acad Sci USA* **80**: 5907–5911, 1983.
4. Rigby W, Shen L, Ball ED, Guyre PM and Fauger MW, Differentiation of a human monocytic cell line by 1,25 dihydroxyvitamin D_3 (calcitriol). A morphologic phenotype and functional analysis. *Blood* **64**: 1110–1115, 1983.
5. Colston KW, Colston MJ and Feldman D, 1,25-dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of growth in culture. *Endocrinology* **108**: 1082–1086, 1981.
6. Frampton RJ, Omond SA and Eisman JA, Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D_3 metabolites. *Cancer Res* **43**: 4443–4447, 1983.
7. Brehier A and Thomasset M, Human colon cell line HT-29. Characterisation of 1,25-dihydroxyvitamin D_3 receptor and induction of differentiation by the hormone. *J Steroid Biochem* **29**: 265–270, 1988.
8. Hosomi J, Hosoi J, Abe E, Suda T and Kuroki T, Regulation of terminal differentiation of cultured mouse epidermal cells by 1α,25-dihydroxyvitamin D_3. *Endocrinology* **113**: 1950–1957, 1983.
9. Koga M, Eisman JA and Sutherland RL, Regulation of epidermal growth factor receptor levels by 1,25-dihydroxyvitamin D_3 in human breast cancer cells. *Cancer Res* **48**: 2734–2739, 1988.
10. Simpson R, Hsu T, Begley DA, Mitchell BS and Alizaden BN, Transcriptional regulation of the c-myc proto-oncogene by 1,25-dihydroxyvitamin D_3 in HL60 promyelocytic leukemia cells. *J Biol Chem* **262**: 41040–41048, 1987.
11. Manglesdorf D, Koeffler HP, Donaldson CA, Pike JW and Haussler MR, 1,25-Dihydroxyvitamin D_3-induced differentiation in a human promyelocytic leukemia cell line (HL60): receptor mediated maturation to macrophage-like cells. *J Cell Biol* **98**: 391–398, 1984.
12. Dokoh S, Donaldson CA and Haussler MR, Influence of 1,25-dihydroxyvitamin D_3 on cultured osteogenic sarcoma cells: connection with the 1,25-dihydroxyvitamin D_3 receptor. *Cancer Res* **44**: 2103–2109, 1984.
13. Eisman JA, Barkla DH and Tutton PJ, Suppression of in vivo growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D3. *Cancer Res* **47**: 21–25, 1987.
14. Honma Y, Hozumi M, Abe E, Konno K, Fukushima M, Hata S, Nishil Y, DeLuca HF and Suda T, 1-25 Dihydroxyvitamin D_3 and 1α hydroxyvitamin D_3 prolong survival time of mice inoculated with myeloid leukaemia cells. *Proc Natl Acad Sci USA* **80**: 201–204, 1983.
15. Colston KW, Berger U and Coombes RC, Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet* **1**: 188–191, 1989.
16. Ostrem VK, Tanaka Y, Prahl J, DeLuca HF and Ikekawa N, 24- and 26-homo-1,25-dihydroxyvitamin

D3: preferential activity in inducing differentiation of human leukemia cells HL-60 *in vitro*. *Proc Natl Acad Sci USA* **84**: 2610–2614, 1987.

17. Abe J, Morikawa M, Miyamoto K, Kaiho S, Fukushima M, Miyaura C, Abe E, Suda T and Nishii Y, Synthetic analogues of vitamin D_3 with an oxygen atom in the side chain skeleton. A trial of the development of vitamin D compounds which exhibit potent differentiation-inducing activity without inducing hypercalcemia. *FEBS Lett* **226**: 58–62, 1987.
18. Binderup L and 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* **37**: 889–895, 1988.
19. Kragballe K, Gjertsen BT, De Hoop D, Karlsmark T, van de Kerkhof PCM, Larko O, Nieboer C, Roed-Petersen J, Strand A and Tikjøb G, Double blind right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. *Lancet* **337**: 193–196, 1991.
20. Bower M, Colston KW, Stein RC, Hedley A, Gazet JC, Ford HT and Coombes RC, Topical calcipotriol treatment in advanced breast cancer. *Lancet* **337**: 701–702, 1991.
21. Colston KW, Chander SK, Mackay AG and Coombes RC, Effects of synthetic Vitamin D analogues on breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem Pharmacol* **44**: 693–702, 1992.
22. Sorenson H, Binderup L, Calverley MJ, Hoffmeyer L and Andersen NR, *In vitro* metabolism of calcipotriol (MC 903), a vitamin D analogue. *Biochem Pharmacol* **39**: 391–393, 1990.
23. Binderup E, Calverley MJ and Binderup L, Synthesis and biological activity of 1α-hydroxylated vitamin D analogues with polyunsaturated side chains. In: *Vitamin D. Gene Regulation, Structure–Function Analysis and Clinical Application* (Eds. Norman AW, Bouillon R and Thomasset M), pp. 192–193. Walter de Gruyer, Berlin, 1991.
24. Colston KW, Wilkinson JR and Coombes RC, 1,25-Dihydroxyvitamin D_3 binding in oestrogen-responsive rat breast tumour. *Endocrinology* **119**: 397–403, 1986.
25. Hauschka PV, Mavrako AE, Iafrati MD, Doleman SE and Klagsbrun M. Growth factors in bone matrix. *J Biol Chem* **261**: 12665–12674, 1986.
26. Wilkinson JR, Williams JC, Singh D, Gross PE, Easton D and Coombes RC, Response of nitrosomethylurea-induced rat mammary tumour to endocrine therapy and comparison with clinical response. *Cancer Res* **46**: 4862–4865, 1986.
27. Deluca HF and Ostrem VK, Analogs of the hormonal form of vitamin D and their possible use in leukemia. *Prog Clin Biol Res* **259**: 41–55, 1988.
28. Zhou JY, Norman AW, Lübbert M, Collins ED, Uskokovic MR and Koeffler HP, Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone mobilization. *Blood* **74**: 82–93, 1989.
29. Ostrem VK, Lau WF, Lee SH, Perlman K, Prahl J, Schnoes HK and DeLuca HF, Induction of monocytic differentiation of HL-60 cells by 1,25-dihydroxyvitamin D analogs. *J Biol Chem* **262**: 14164–14171, 1987.
30. Shiina Y, Abe E, Miyaura C, Tanaka H, Yamada S, Ohnori M, Nakayama K, Takayama H, Nishii Y and DeLuca HF, Biological activity of 24,24 difluoro-1α,25-dihydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3-26,23-lactone in inducing differentiation of human myeloid leukaemia cells. *Arch Biochem Biophys* **220**: 90–94, 1983.
31. Abe J, Nakawo T, Nishii Y, Matsumoto T, Ogata E and Ikeda K, A novel vitamin D_3 analogue 22-oxa 1,25-dihydroxyvitamin D_3 inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcaemia. *Endocrinology* **129**: 832–837, 1991.
32. Perlman K, Kutner A, Prahl J, Smith C, Inaba M, Schnoes HK and DeLuca HF, 24-Homologated 1,25-dihydroxyvitamin D_3 compounds: separation of calcium and cell differentiation activities. *Biochemistry* **29**: 190–196, 1990.
33. Norman AW, Zhou JY, Henry HL, Uskokovic MR and Koeffler HP, Structure–function studies on analogues of 1 alpha,25-dihydroxyvitamin D_3: differential effects on leukemic cell growth, differentiation, and intestinal calcium absorption. *Cancer Res* **50**: 6857–6864, 1990.
34. Kragballe K and Wildfang I, Calcipotriol (MC903), a novel vitamin D_3 analogue stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes. *Dermatol Res* **282**: 164–167, 1990.
35. Freake H, Abayasakera G, Iwasaki J, Marcocci C, MacIntyre I, McClelland R, Skilton R, Easton D and Coombes RC, Measurement of 1,25-dihydroxyvitamin D_3 receptors in breast cancer and their relationship to biochemical and clinical indices. *Cancer Res* **44**: 1677–1681, 1984.
36. Eisman J, Suva L, Sher E, Pierce P, Funder J and Martin T J, 1,25-Dihydroxyvitamin D_3 receptor in human breast cancer. *Cancer Res* **41**: 5121–5124, 1981.
37. Berger U, Wilson P, McClelland R, Colston K, Haussler MR, Pike JW and Coombes RC, Immunocytochemical detection of 1,25-dihydroxyvitamin D_3 receptor in breast cancer. *Cancer Res* **47**: 6793–6799, 1987.
38. Binderup L, Latini SL, Binderup E, Bretting C, Calverley M and Hansen K, 20-Epi-vitamin D_3 analogues: a novel class of potent regulators of cell growth and immune responses. *Biochem Pharmacol* **42**: 1569–1575, 1991.
39. Binderup L, Latini S and Kissmeyer A-M, New vitamin D analogues with potent effects on cell growth regulation and immune responses: structure–activity studies. In: *Vitamin D. Gene Regulation, Structure-function Analysis and Clinical Application* (Eds. Norman AW, Bouillon R and Thomasset M), pp. 478–485. Walter de Gruyter, Berlin, 1991.
40. Baker AR, McDonnell D, Hughes MR, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J and O'Malley BW, Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci USA* **85**: 3294–3298, 1988.
41. Haussler MR, Mangelsdorf DJ, Komm BS, Terpening CM, Yamaoka K, Allegretto EA, Baker AR, Shine J, McDonnell DP and Hughes M, Molecular biology of the vitamin D hormone. *Recent Prog Hormone Res* **44**: 263–305, 1988.
42. Pike JW, Sleator NM and Haussler MR, Chicken intestinal receptor for 1,25-dihydroxyvitamin D3. Immunologic characterization and homogeneous isolation of a 60,000-dalton protein. *J Biol Chem* **262**: 1305–1311, 1987.
43. Naveh-Many T, Marx R, Keshet E, Pike JW and Silver J, Regulation of 1,25-dihydroxyvitamin D_3 receptor gene expression by 1,25-dihydroxyvitamin D_3 in the parathyroid *in vivo*. *J Clin Invest* **86**: 1968–1975, 1990.