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EB1089, a synthetic analogue of vitamin D, induces apoptosis in breast cancer cells *in vivo* and *in vitro*

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1 Effects of the synthetic vitamin D analogue EB1089 on indices of apoptosis in cultured human breast cancer cells and in nitrosomethylurea-induced rat mammary tumours *in vivo* were investigated.

2 At a dose of 0.5 μg kg^{-1} body weight, EB1089 caused significant inhibition of tumour progression over the 28 day treatment period in the absence of a significant increase in serum calcium concentration. Higher doses of EB1089 (1 and 2.5 μg kg^{-1}) produced substantial regression of the experimental tumours which was accompanied by a striking change in the histological appearance of tumours consistent with induction of tumour cell death.

3 Fragmentation of genomic DNA is a characteristic feature of apoptosis. With the terminal transferase (TdT) assay, 3′ DNA breaks indicative of DNA fragmentation were detected histochemically in mammary tumour cells from animals treated with EB1089 (2.5 μg kg^{-1}) for 14 days.

4 Effects of the vitamin D analogue on induction of apoptosis were examined *in vitro* using the MCF-7 human breast cancer cell line. Using the TUNEL method, positive nuclear staining indicative of DNA fragmentation was detected in cells treated for 4 days with 10 nM EB1089. Apoptosis was also quantitated using a cell death ELISA which revealed a time and dose dependent induction of apoptosis by EB1089.

5 The effects of EB1089 on the expression of two oncoproteins which may regulate apoptosis, bcl-2 and bax were examined by Western analysis. In MCF-7 cell cultures treated with 1,25$(OH)_2D_3$ or EB1089 (1×10^{-8} M), bcl-2 protein levels were decreased in a time-dependent manner relative to control levels. In contrast bax protein was not markedly regulated by these compounds. Densitometric analyses indicate that the vitamin D compounds lower the bcl-2/bax ratio favouring increased susceptibility of MCF-7 cells to undergo apoptosis.

6 These results suggest that the synthetic vitamin D analogue EB1089 may promote tumour regression by inducing active cell death.

Keywords: Vitamin D analogues; apoptosis; oncoproteins; DNA fragmentation; breast cancer

Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) has been shown to possess many properties unrelated to its classical functions in the control of bone and mineral metabolism. Notably it is a potent inducer of cell differentiation in a number of established cancer cell lines. In tumour bearing animals, 1,25$(OH)_2D_3$ and its syn-thetic analogue 1α-hydroxyvitamin D_3, which undergoes conversion to 1,25$(OH)_2D_3$ *in vivo* suppress tumour growth, inhibit metastasis and prolong survival (Honma *et al*., 1983; Colston *et al*., 1992a). Although the therapeutic potential of 1,25$(OH)_2D_3$ seems encouraging, the clinical use of this compound is limited by its potent calcaemic activity.

Administration of more than a few micrograms per day leads to hypercalcaemia (Koeffler *et al*., 1985). To combat these unwanted side effects, newer vitamin D analogues have been synthesized which retain the ability to control cell proliferation and differentiation but display reduced calcaemic activity. Recent studies have demonstrated that certain of these synthetic vitamin D analogues can cause regression of experimental mammary tumours, as well as marked inhibition of oestrogen receptor positive and oestrogen-receptor negative breast cancer cell proliferation (Abe *et al*., 1991; Colston *et al*., 1992a,b). We have carried out preclinical trials with a range of structurally modified compounds to assess effects on tumour growth *in vivo* using a rat model of hormone dependent breast cancer in which mammary tumours are induced with the carcinogen nitrosomethylurea (NMU). Activity profiles of a series of analogues with modification or elongation of the C17 side chain have been assessed. Certain of these compounds, and in particular the analogue EB1089, cause regression of these carcinogen induced mammary tumours (Colston *et al*., 1995). However, the precise mechanism of action of these new compounds remains unclear.

Tumour regression occurs when the rate of cell death is greater than the rate of cell proliferation. Apoptosis (programmed or active cell death) is an inherent, energy dependent process present within cells, whereby a distinct series of biochemical and molecular events leads to the death of cells by specific signals (Wyllie, 1987; Williams, 1991). A number of genes and proteins are implicated in the regulation of apoptosis, which include the tumour suppressor gene, p53 (Lane, 1992; Gottlieb & Oren, 1996), the caspases (Jacobson & Evan, 1994), the APO-1/Fas antigen (Cosman, 1994) and the bcl-2 family (Yang *et al*., 1996). Aberrant expression and/or activation of these specific genes and proteins may result in increased resistance or susceptibility to apoptosis. For example, the bcl-2 gene product confers resistance to active cell death induced by a number of stimuli (Reed, 1994), whilst its homologue, bax promotes apoptosis (Oltvai *et al*., 1993). The ability of bcl-2 and bax to form heterodimers suggests that the susceptibility of a cell to undergo apoptosis appears to

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depend in part on the ratio of bcl-2 to bax, with a lower ratio favouring active cell death. Studies indicate that reduced expression of bax is associated with poor response of patients with metastatic breast cancer to chemotherapy (Krajewski *et al*., 1995).

Our previous studies and those of others have demonstrated the ability of $1,25(OH)_2D_3$ and its analogues to differentially regulate apoptosis-related gene products such as bcl-2 and p53 in breast cancer cell lines so as to promote this process (Elstner *et al*., 1995; James *et al*., 1995, 1996; Simboli-Campbell *et al*., 1997). In the present study we have examined effects of EB1089 on indices of apoptosis in mammary tumours *in vivo* and in cultured human breast cancer cells.

Methods

Compounds

$1,25(OH)_2D_3$ and the vitamin D analogue EB1089 (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) were provided by Department of Chemistry, Leo Pharmaceutical Products, Ballerup, Denmark.

Experimental animals

Oestrogen sensitive mammary tumours were induced in female virgin inbred Ludwig/Wistar/Olac rats (Olac Ltd., Oxon, U.K.) by the method described by Williams *et al*. (1981). Briefly, 50 day old female rats were given 50 mg nitrosomethylurea (NMU; Sigma Chemical Co., Poole, Dorset, U.K.) subcutaneously into the right flank. Two further injections were similarly given 14 and 28 days later. The rats were then transferred to the Biological Research Facility, St George's Hospital Medical School, where they were kept at 22–23°C with a 12 h light period per day and fed Ludwig high fat diet (Special Diet Services Ltd., Witham, U.K.). After 20 weeks, 70–80% of the animals developed mammary tumours.

In vivo *treatment with EB1089*

Rats bearing at least one assessable tumour (>10 mm in diameter) were randomly assigned to treated or control groups. EB1089 was given in propylene glycol p.o. daily at 0.5, 1.0 or 2.5 μg kg^{-1} for 14 or 28 days. These doses of EB1089 were chosen on the basis of studies carried out to determine the calcaemic effects of this compound *in vivo* (Mathiasen *et al*., 1993). Tumour volume was assessed weekly. Twenty-four hours prior to the end of the treatment period, rats were placed in individual metabolic cages and urine was collected. At the end of the experiment, animals were weighed and exsanguinated under halothane anaesthesia. Tumours were excised, immediately frozen in liquid nitrogen and stored at −70°C. Serum was stored at −20°C until analysed.

Measurement of tumour volume

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 $V = 1/6\pi[(D1 \times D2)^{3/2}]$ (where D1 and D2 are the two diameters). The percentage change in tumour volume compared with the tumour volume at the start of treatment was calculated for each rat. Animals whose tumours showed signs of ulceration or in which tumour burden became excessive were culled. The percentage change in tumour volume at each week was compared between treated and respective control groups using the non-parametric Mann-Whitney *U*-test.

Cells and cell culture

MCF-7 breast cancer cells were routinely maintained in Dulbecco's modification of Eagle's essential medium (DMEM), supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 5% foetal calf serum (Life Technologies, Paisley, U.K.). For studies of bcl-2 and bax expression, cells (1×10^5) were seeded into 75 cm^2 flasks and cultured for 2 days before being dosed with the desired concentration of test compound for up to 6 days.

Western blot analysis

MCF-7 cell cultures were harvested and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0, with 0.1% Triton X-100, 0.01 mg ml^{-1} aprotinin and 0.05 mg ml^{-1} PMSF, Sigma, Poole, U.K.). Equivalent protein extracts (25 μg) from each sample were electrophoresed on 10% SDS–PAGE gels. Total protein was quantitated by the method of Bradford (1976) and equivalent loading was confirmed by Coomassie blue staining of replicate gels. Proteins were transferred onto Hybond C-Super nitrocellulose (Amersham International, U.K.) in a Bio-Rad Trans Blot apparatus. Nitrocellulose matrices were preblocked with 5% non-fat milk powder in PBS and 0.05% Tween 20 for 1 h at room temperature. Following PBS/Tween washes, preblocked matrices were incubated with 2 μg ml^{-1} equivalent of mouse monoclonal antibodies against bcl-2 or rabbit polyclonal antibodies to bax (Santa Cruz, Heidelberg, Germany). Bcl-2 and bax were detected by horseradish peroxidase-conjugated secondary antibodies to anti-mouse and anti-rabbit immunoglobulins respectively (Amersham International, U.K.) for a 1 h incubation at room temperature and visualizing specific bands by enhanced chemiluminescence (ECL, Amersham International, U.K.). Densitometry was performed on a Microtek Scanmaker IISP flat bed scanner and quantitated with NIH Image 1.52 software. Statistical significance was evaluated using ANOVA with a Fisher's PLSD post test (Statview 4.5, Abacus Concepts, Berkeley, California, U.S.A.).

Identification of DNA fragmentation

Frozen sections of mammary tumours from rats treated for 14 days with 2.5 μg kg^{-1} body weight EB1089 were fixed in 4% formaldehyde in PBS. In addition, MCF-7 cells were grown in chamber slides (Life Technologies, Paisley, U.K.) for 4 days in the presence or absence of test substances. The cells were then fixed in 4% formaldehyde in PBS and washed in PBS before assay. DNA fragmentation within cell nuclei was detected using the ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, Maryland, U.S.A.) according to the manufacturers' instructions. Briefly, cells or tissue sections were incubated for 1 h with digoxigenin-dUTP and terminal transferase (TdT), the latter linking digoxigenin-dUTP to 3′-OH fragmented ends of DNA. Following a wash to stop the primary incubation, the cells were then incubated for 30 min with a digoxigenin antibody conjugated to horseradish peroxidase. Visualization of apoptotic cells was achieved by chromogenic staining with diaminobenzidine (DAB).

Quantitation of active cell death

MCF-7 cells were seeded at a density of 1×10^4 cells ml^{-1} in six well plates in DMEM supplemented with 2.5% FCS. Graded concentrations of EB1089 were added 3 h after seeding and fresh dilutions were added every second day when the medium was changed for up to 6 days. Apoptosis was measured by the Cell Death Detection ELISA™ (Boehringer Mannheim, U.K.) according to the manufacturers' instructions. This assay quuantitates cytoplasmic histone-associated DNA fragments. Results were normalized by directly counting trypan blue excluding cells and expressed in relation to cultures treated with ethanol vehicle. At least two separate experiments, each with two replicate estimations, were performed.

Results

Changes in tumour volume

Effects of EB1089 on the growth of NMU-induced rat mammary tumours and serum calcium concentration are given in Figure 1. Tumour-bearing rats were treated with 0.5, 1.0 or 2.5 $\mu g\ kg^{-1}$ EB1089 p.o. daily for 28 days. Figure 1a shows change in tumour volume (expressed as percentage of initial tumour volume). Mean serum calcium concentrations at the end of the treatment period for each treatment group are shown in Figure 1b. At a dose of 0.5 $\mu g\ kg^{-1}$ body weight p.o. daily for 28 days EB1089 caused significant inhibition of tumour progression in the absence of a significant rise in serum calcium concentration. Higher doses of the analogue (1.0 and 2.5 $\mu g\ kg^{-1}$ daily) produced tumour regression although significant increases in serum calcium concentrations were observed. In a second series of experiments, rats were treated daily for 14 or 28 days with 2.5 $\mu g\ kg^{-1}$ and changes in tumour growth, serum calcium concentration and urinary calcium excretion were determined. Tumour tissue was obtained from rats treated for 14 days and 28 days of treatment. Figure 2 shows the growth of tumours in control animals and rats treated with 2.5 $\mu g\ kg^{-1}$ EB1089 over a 28 day period. The analogue caused substantial tumour regression such that at the end of the 28 day treatment period mean tumour volume was only 20% of initial value and significant inhibition of tumour progression was observed after 14 days treatment. Table 1a illustrates the effects of 2.5 $\mu g\ kg^{-1}$ EB1089 on the number of animals showing tumour regression and tumour development during the 28 day treatment period. Of the 12 animals in the group treated with 2.5 $\mu g\ kg^{-1}$ EB1089, 11 showed reduction of total tumour volume of more than 50% at 28 days of treatment. No control animals displayed tumour regression. Furthermore, while the number of initial tumours was similar in each group

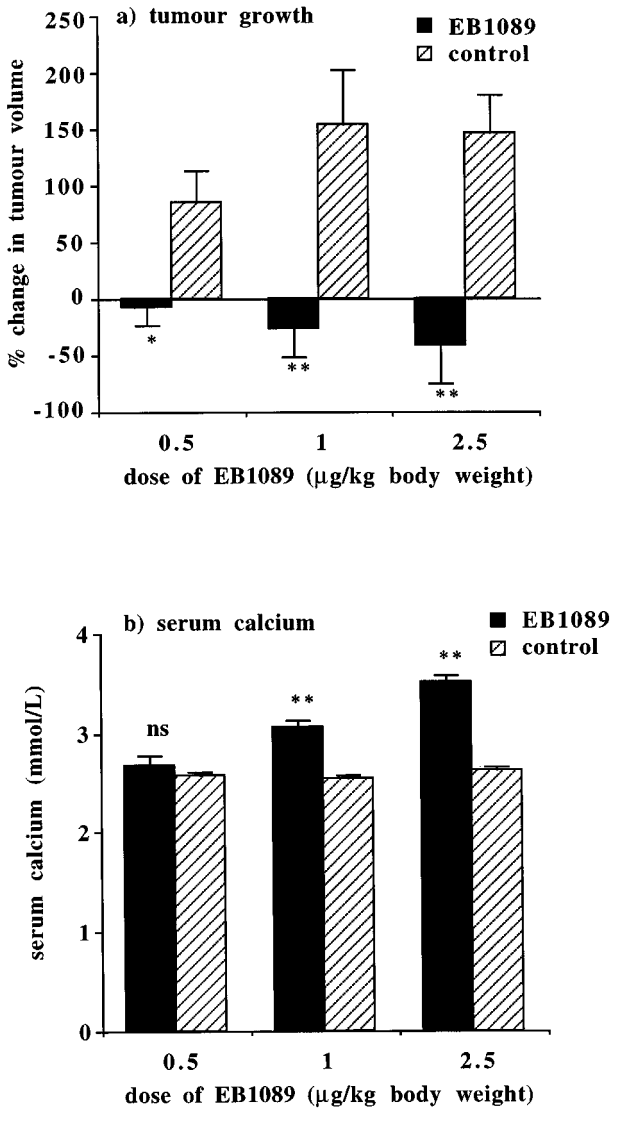

Figure 1 Effects of increasing doses of EB1089 on the growth of NMU-induced rat mammary tumours and serum calcium concentration. (a) Tumour-bearing rats were treated with 0.5, 1.0, 2.5 $\mu g\ kg^{-1}$ body weight with EB1089 p.o. daily for 28 days. Percentage change in tumour volume in control animals from day 0 and are shown as means ± s.e.mean (hatched bars). Negative values indicate tumour regression in EB1089 treated animals (solid bars). (b) Serum calcium concentration in EB1089 treated animals (solid bars) and control animals (hatched bars) at the end of the treatment period. $*P<0.01$, $**P<0.005$ from respective controls.

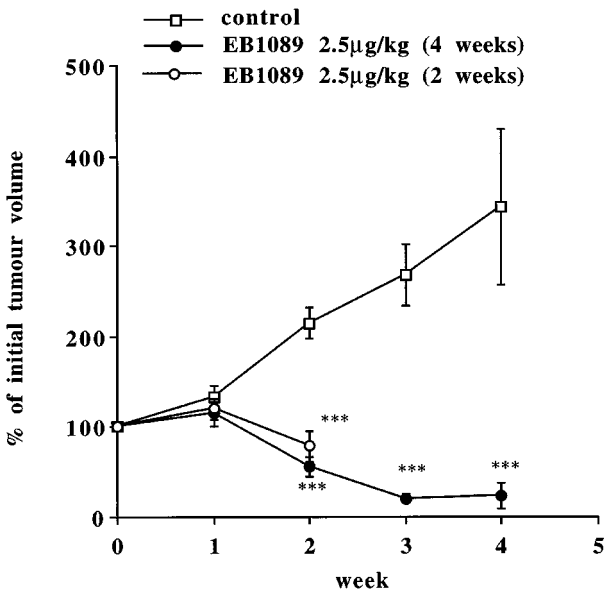

Figure 2 Effect on tumour growth of daily oral treatment with EB1089 (2.5 $\mu g\ kg^{-1}$ body weight for 2 weeks or 4 weeks. Control animals received vehicle alone. Results are expressed as % of initial tumour volume (100%) and are shown as means ± s.e.mean. Statistical comparisons were made using the Mann-Whitney *U*-test, $***P<0.001$. Serum and urinary calcium levels are shown in Table 1b.

(15 in controls vs 17 in treated group), 13 new tumours developed in the control group during the 28 day treatment period, but no new tumours developed in the EB1089 treated animals, indicating that the vitamin D analogue may also be effective in preventing tumour development. A significant increase in serum calcium concentration was already evident at 14 days of treatment and changes in urinary calcium excretion could be detected after treatment for 7 days (Table 1b).

Histology of tumours and in situ *detection of DNA fragmentation*

Figure 3 demonstrates the histological appearance of control tumours and tumours obtained from rats treated with 2.5 μg kg^{-1} EB1089 for 28 days. The majority of tumours induced by NMU are classified as adenocarcinomas, based upon cytological abnormalities and growth pattern exhibiting a papillary pattern with many mitoses (Stubbs *et al*., 1990;

Table 1 (a) Effects of EB1089 (2.5 μg kg^{-1} body weight p.o. daily for 28 days) on tumour progression and development

Treatment	*Number of rats*	*Number of tumours initial*	*new*	*Regression* >50%	<50%	*Progression*	*Response rate*$^{+}$	P *value*$^{\wedge}$
Control	12	15	13	0	0	12	0	—
EB1089	10	17	0	8	1	1	80%	<0.001

$^{+}$response rate (%) = $\frac{\text{No. of animals with greater than 50\% tumour regression} \times 100}{\text{total no. of animals}}$

$^{\wedge}$Statistical comparisons were made using the non-parametric Mann-Whitney *U*-test.

(b) Changes in serum calcium and urinary calcium excretion throughout the treatment period

	Serum calcium mmol l^{-1}	*Urinary calcium* μmol 24 h^{-1}
Control	2.74±0.04	65.5±8.9
1 week EB1089	nd	273.1±34.5***
2 weeks EB1089	3.25±0.33***	255.3±39.8***
4 weeks EB1089	3.38±0.06***	262.2±36.0***

***$P<0.001$.

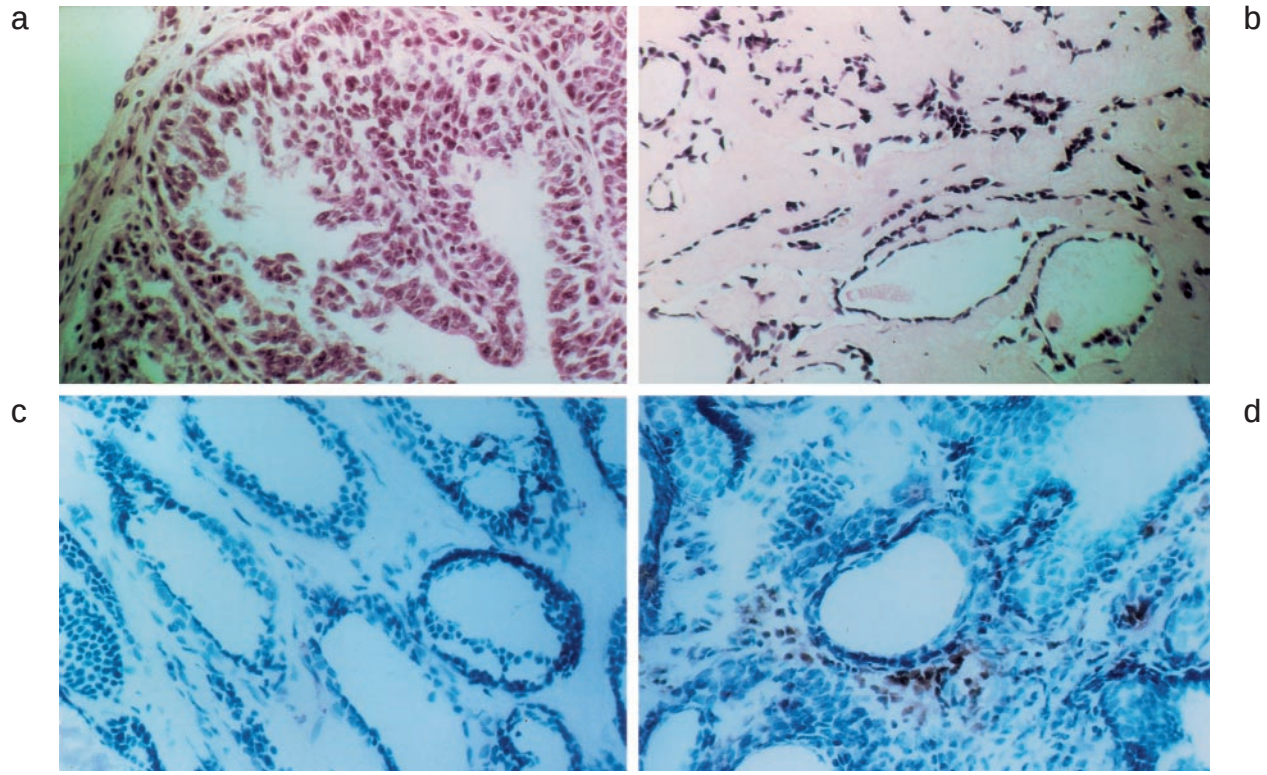

Figure 3 (a and b) Histology of mammary tumour sections (H&E stain). Control (a) and EB1089 treated rats (b) after 28 days of treatment (original magnification, ×40). (c and d) *In situ* detection of DNA fragmentation, a key feature of apoptosis. Effects of EB1089 on induction of DNA fragmentation in mammary tumour cells. Sections of control tumour (c) and tumour sectioned after 14 days treatment with 2.5 μg kg^{-1} EB1089 p.o. daily (d). Note increased reactivity in tumour from EB1089 treated rat (original magnification, ×40).

Mackay *et al*., 1996). In contrast, sections of tumours from animals treated with EB1089 showed marked loss of cellularity (Figure 3b). Epithelia were mostly monolaminate and nuclei pale staining. Mitotic figures were hard to find. These marked histological changes indicate that induction of apoptosis may be a feature of the anti-tumour effects displayed by the vitamin D analogue.

During apoptosis, Ca^{2+} and Mg^{2+} dependent endonuclease activity produces DNA fragments that are multimers of about 180 bp nucleosomal units (Arends *et al*., 1990). We have employed the TUNEL method to detect evidence of DNA fragmentation in nuclei of tumour cells from control animals and rats treated for 14 days with 2.5 μg kg^{-1} EB1089. Using this method, residues of digoxigenin-nucleotide are catalytically added to the multitude of 3′-OH DNA ends generated by this fragmentation by the terminal deoxynucleotidyl transferase reaction (Schmitz *et al*., 1991). Incorporated nucleotides can then be detected by peroxidase-conjugated anti-digoxigenin antibody. Figure 3 shows the effects of EB1089 treatment on induction of DNA fragmentation in mammary tumour cells and compares the appearance of cryosections of control tumour (Figure 3c) with tumor sectioned after 14 days treatment with 2.5 μg kg^{-1} EB1089 p.o. daily (Figure 3d). Following staining by the TUNEL method, little staining was noted in control tumours, whereas cryosections from treated rat tumours demonstrated considerable nuclear reactivity, indicating the presence of DNA fragmentation. These findings demonstrate that specific DNA fragmentation which is associated with the process of apoptosis can be demonstrated in sections of mammary tumours which are regressing in response to treatment with a vitamin D analogue.

Induction of apoptosis in vitro

In order to further characterize effects of vitamin D derivatives on induction of apoptosis in breast cancer cells, we have utilized the oestrogen responsive MCF-7 breast cancer cell line to examine effects *in vitro*. The terminal transferase reaction was used to identify cell nuclei containing fragmented DNA in MCF-7 cells incubated in the presence of $1,25(OH)_2D_3$ or EB1089 (1×10^{-8} M for 4 days), (Figure 4). As a positive control for induction of apoptosis, cultured cells which had been subjected to u.v. irradiation prior to fixation were used. Results showed little staining reaction in cell cultures which received ethanol vehicle alone (average $\leqslant 1$ positive nuclei per field, Figure 4a). Cells which received 1×10^{-8} M $1,25(OH)_2D_3$ or EB1089 showed positively reacting nuclei in a proportion of cells (average 8 and 15 per field respectively. Figure 4b and c).

Quantitation of apoptosis

Quantitative assessment of the ability of EB1089 to induce apoptosis was determined using the cell death ELISA. MCF-7 cells were treated for 2, 4 and 6 days with 1×10^{-7} M EB1089. Figure 5a illustrates that over this time period, dramatic increases in oligonucleosome enrichment were observed, with a 12 fold induction of apoptosis occurring following 6 days treatment. Lower concentrations of the analogue (1×10^{-8} M and 1×10^{-9} M) also showed comparable induction of active cell death after 6 days treatment (Figure 5b).

Effects of vitamin D derivatives on expression of bcl-2 and bax

The regulation of bcl-2 and bax oncoprotein expression by $1,25(OH)_2D_3$ and EB1089 were assessed by Western analysis. Figure 6a and b depict the regulation of bcl-2 and bax protein levels respectively by 1×10^{-8} M $1,25(OH)_2D_3$ or EB1089 over a time period of 2, 4 and 6 days. A progressive reduction of bcl-2 protein was observed with EB1089 treatment over the time course, such that at 6 days treatment the analogue down-regulated bcl-2 protein by 80% relative to control (as evaluated by densitometric analysis). In contrast, bax protein levels were modestly up-regulated by the vitamin D compounds (Figure 6a and corresponding densitometry). Figure 6b shows composite densitometric results from four observations of the bcl-2/bax ratio present following treatment with the vitamin D derivatives. EB1089 reduced bcl-2/bax levels such that by 6 days treatment, the analogue had decreased bcl-2/bax protein expression by 41% relative to control.

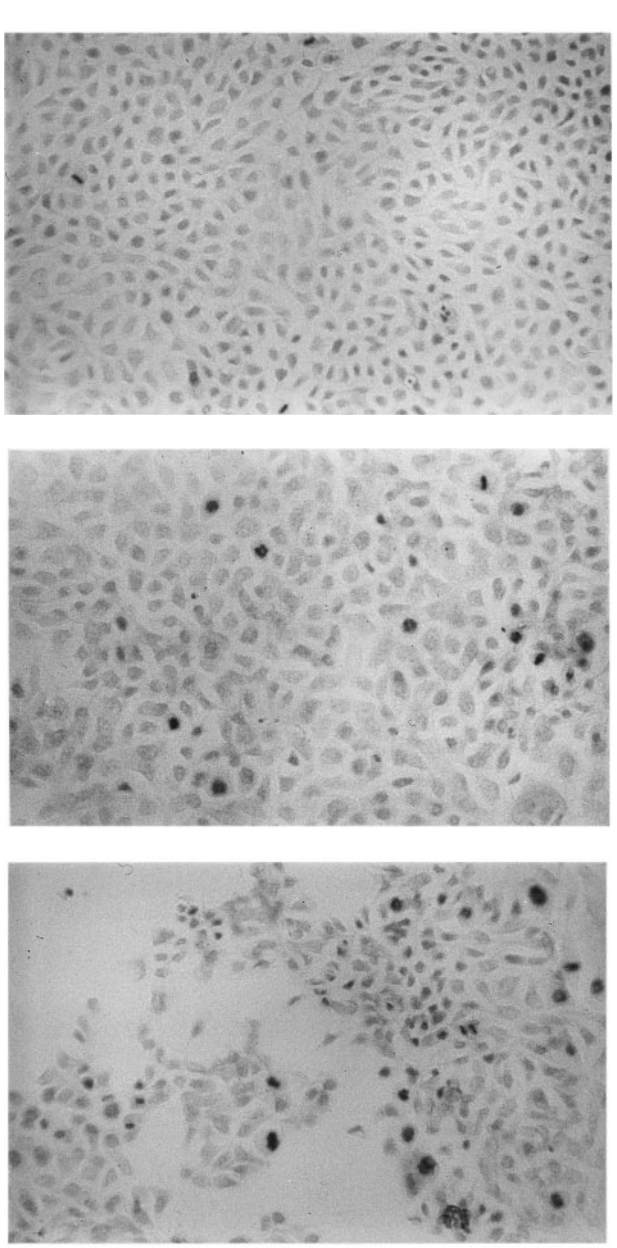

Figure 4 Effects of EB1089 on induction of DNA fragmentation in MCF-7 human breast cancer cells. Cells were cultured in the presence or absence of 10 nM $1,25(OH)_2D_3$ or EB1089 before fixation and detection of apoptotic nuclei by the TdT reaction. (a) Ethanol vehicle, (b) $1,25(OH)_2D_3$, (c) EB1089 treated cells (original magnification, $\times 40$).

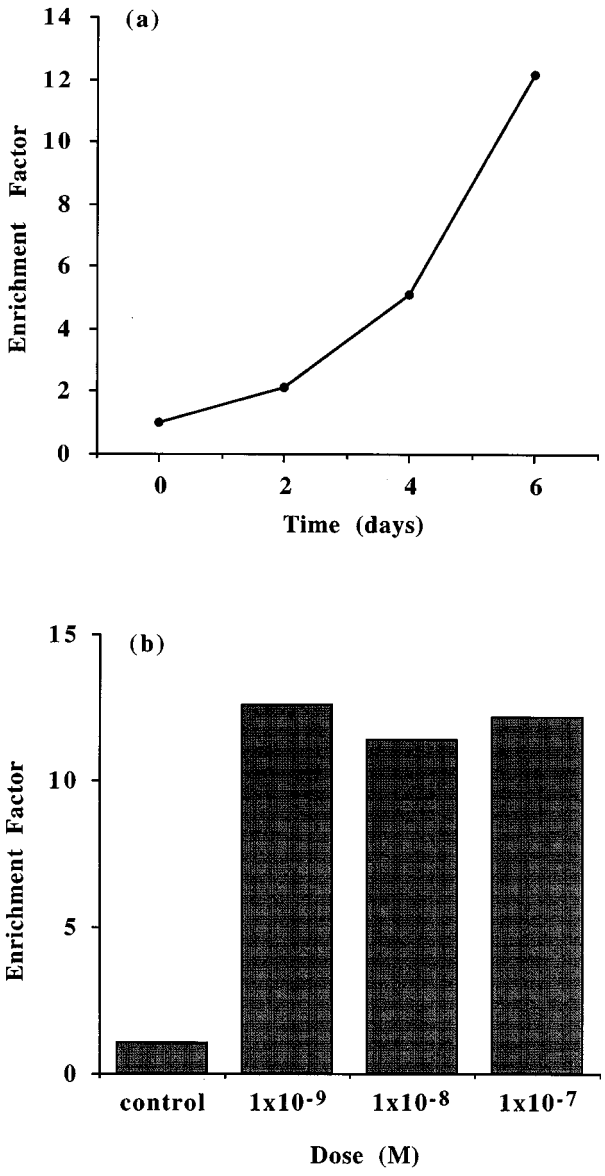

Figure 5 Detection of cytoplasmic oligonucleosomes in MCF-7 cells exposed to vitamin D derivatives. (a) MCF-7 cell cultures were treated for 2, 4 and 6 days with either ethanol vehicle or EB1089 (1×10^{-7} M). (b) Effect of EB1089 ($1 \times 10^{-7} - 1 \times 10^{-9}$ M) on oligonucleosome enrichment following 6 days treatment. Following correction for cell number, cells were lysed and assessed for the presence of oligonucleosomes by ELISA according to the manufacturers' protocol. The enrichment factor represents the ratio between the absorbances of the dying/dead cells and viable cells.

Discussion

Apoptosis may be induced in endocrine responsive tissues and tumours following removal of the trophic hormone. The process occurs naturally in the mammary gland after weaning, and hormone dependent mammary tumours and breast cancer cells undergo apoptosis following endocrine ablation or anti-oestrogen treatment (Kyprianou *et al*., 1991; Warri *et al*., 1993). The findings from the present study demonstrate that the synthetic vitamin D analogue EB1089, which displays reduced calcaemic activity relative to $1,25(OH)_2D_3$, induces apoptosis in breast cancer cells *in vivo* and *in vitro*. The highest dose of EB1089 tested, 2.5 μg kg^{-1} body weight daily, produced substantial regression of the experimental mammary tumours, which was accompanied by a striking change in histological appearance consistent with induction of tumour cell death. Concomitantly, DNA fragmentation, a key feature of apoptosis, was detected in tumour cells in cryostat sections of mammary tumours from animals treated with EB1089 using the terminal transferase reaction. Similar *in vivo* results of EB1089 inducing the regression of MCF-7 xenograft tumours in nude mice, and the presence of apoptotic cells in tumour sections over a treatment period of 5 weeks have been reported (VanWeelden & Welsh, 1997; VanWeelden *et al*., 1998).

Previous *in vivo* studies have shown that EB1089 has reduced calcaemic actions compared with $1,25(OH)_2D_3$, the native hormone (Mathiasen *et al*., 1993). In the NMU induced mammary tumour model, inhibition of tumour progression was seen with 0.5 μg kg^{-1} body weight EB1089 in the absence of a significant rise in serum calcium concentration. This same dose of $1,25(OH)_2D_3$ has been shown to cause hypercalcaemia with no significant effect on tumour growth (Colston *et al*., 1992b). Thus EB1089 displays enhanced anti-tumour activity and reduced calcaemic activity relative to $1,25(OH)_2D_3$. A 5 fold higher dose of EB1089 caused striking tumour regression although serum calcium was significantly elevated by 14 days of treatment in conjunction with increased urinary calcium excretion. Preliminary studies indicate that, in the NMU model, calcaemic effects of EB1089 are mediated *via* increased intestinal absorption rather than promotion of bone resorption, since co-treatment with bisphosphonates such as sodium pamidronate did not prevent the elevation of serum calcium concentration in response to 2.5 μg kg^{-1} EB1089 (our unpublished observations).

To further investigate the potential mechanism of action of the vitamin D analogue, we have assessed indices of apoptosis *in vitro* in MCF-7 cells. In these studies we observed striking induction of apoptosis both by visualization of apoptotic cells *in situ* and enrichment of cytoplasmic oligonucleosomes by cell death ELISA. Taken together our results suggest that this vitamin D analogue may cause tumour regression in part by inducing active cell death.

Approximately 80% of breast tumours express vitamin D receptor (VDR), and the presence of the receptor is correlated with prolonged disease free survival time (Berger *et al*., 1991). ER positivity of breast tumours has similarly been associated with increased disease free survival as well as being a predictor of response to endocrine therapy (Mansi & Smith, 1989). Previous studies have indicated that vitamin D derivatives are capable of attenuating the mitogenic effects of oestradiol by down-regulating ER (James *et al*., 1994; Simboli-Campbell *et al*., 1997). However, anti-proliferative effects of the vitamin D compounds are also observed in ER negative cell lines (James *et al*., 1994; Love-Schimenti *et al*., 1996; Flanagan *et al*., 1997), suggesting that modulation of the ER signalling pathway may not be an exclusive mechanism through which the observed actions of these compounds are mediated. Flanagan and colleagues (1997) have demonstrated the ability of $1,25(OH)_2D_3$ and EB1089 to inhibit the growth and induce apoptosis of the ER negative breast cancer cell line, SUM-159PT *in vitro* and *in vivo*. SUM-159PT cells inoculated into ovariectomized nude mice were inhibited in their growth by 80–85% with daily pellet release of 60–120 pmol EB1089 after 28 days, and inspection of excised tumours revealed morphologically distinct apoptotic cells. Interestingly, characterization of a variant MCF-7 cell line resistant to $1,25(OH)_2D_3$ and its analogues revealed that these compounds were unable to inhibit cell growth or promote apoptosis, whilst the anti-proliferative and apoptosis-inducing effects of anti-oestrogens were retained (Narvaez *et al*., 1996). The vitamin D

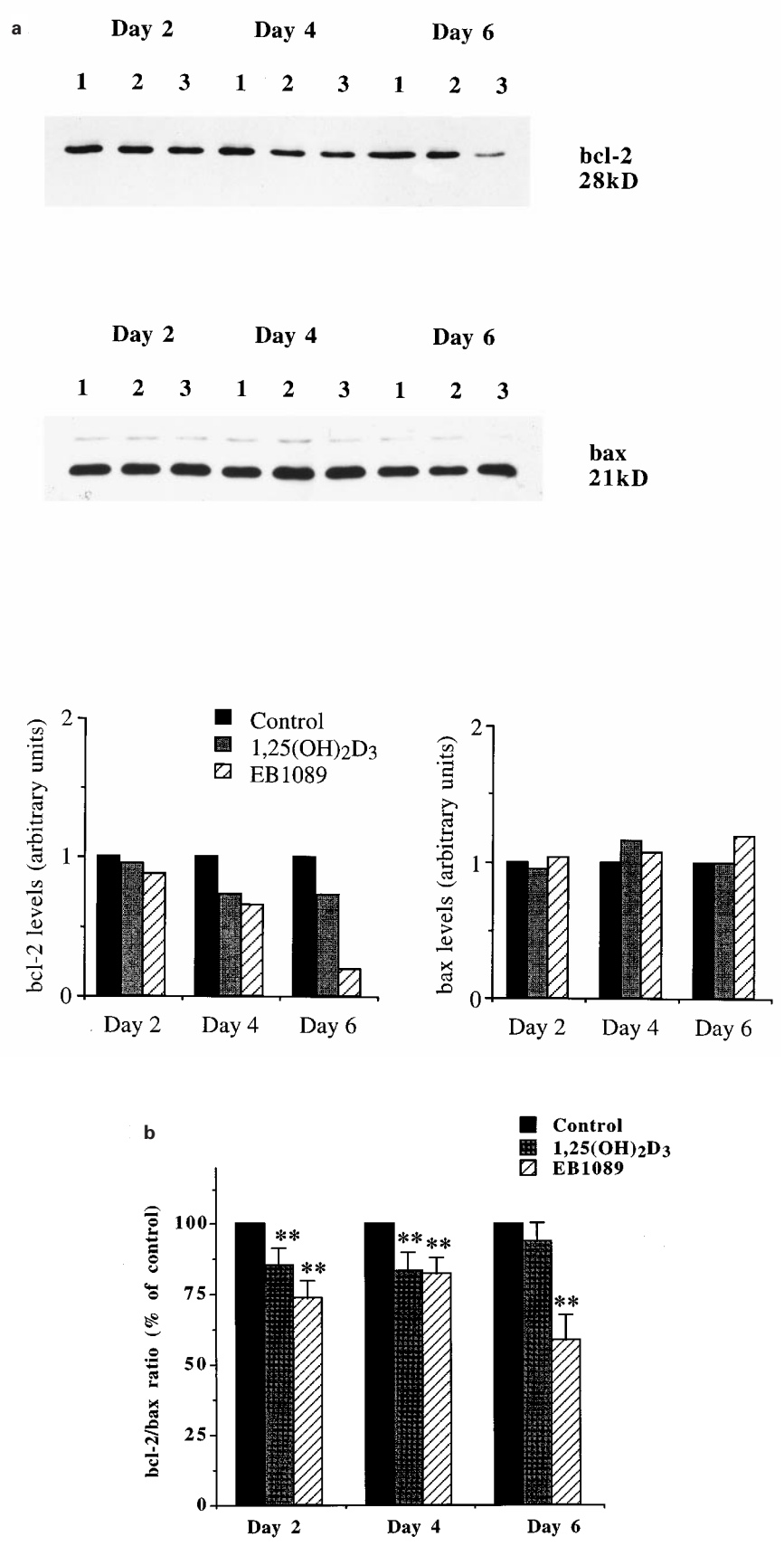

Figure 6 Effects of $1,25(OH)_2D_3$ and EB1089 on expression of bcl-2 and bax. (a) MCF-7 cells were treated for 2, 4 and 6 days with $1,25(OH)_2D_3$ or EB1089 (1×10^{-8} M) before cell extracts were subjected to Western analysis. Lane order: (1) Control, (2) $1,25(OH)_2D_3$, (3) EB1089. Corresponding densitometric analysis for bcl-2 and bax protein levels are shown, with values being expressed relative to control (standardized as 1). (b) Densitometric analysis of bcl-2 and bax levels expressed as bcl-2/bax ratios (% of control, $n = 4 \pm$ s.e.mean). $^{**}P < 0.005$, significant from control (ANOVA).

resistant MCF-7 cells did, however, possess functional VDR, suggesting that VDR signalling has in some way become blocked or dissociated from growth regulatory and apoptotic pathways.

Bcl-2 and its homologues are regarded as important regulators of the apoptotic process, and so their differential expression in certain cell systems may contribute in part to either the survival or the demise of affected cells. In the present study we have demonstrated that the ratio of bcl-2 and bax protein in MCF-7 cells is decreased with $1,25(OH)_2D_3$ and in particular, EB1089 treatment, suggesting alterations in the heterodimerization of these proteins which may promote apoptosis. Several research groups have reported a positive correlation of bcl-2 expression and ER status (Elledge *et al*., 1997; Hori *et al*., 1997), with one report stating that 80% of bcl-2 positive breast tumours were also ER positive and implicating bcl-2 as an ER regulated gene (Leek *et al*., 1994). Other studies have revealed that oestradiol increases bcl-2, but not bax transcript levels in MCF-7 cells, and protects them from undergoing apoptosis (Wang & Phang, 1995). In addition the bcl-2/bax ratio is increased by oestradiol, and associated with the inability of taxol to induce active cell death in MCF-7 cells (Huang *et al*., 1997). Furthermore, wild type p53 has been shown to negatively regulate ER signalling by interacting with the receptor and repressing transcriptional activity mediated by oestradiol (Yu *et al*., 1997). In terms of ER-negative cells we have not observed any marked changes in bcl-2 expression following treatment of MDA-MB-231 cells with 1×10^{-8} M EB1089 for 4 days (our unpublished observations). However, another vitamin D analogue, KH1060 was capable of down-regulating bcl-2 in MDA-MB-231 cells, although no apoptosis was apparent (Elstner *et al*., 1995). These studies highlight that the down-regulation of bcl-2 by vitamin D compounds in certain cell systems may result in anti-proliferative effects independent of induction of apoptosis and that additional pro-apoptotic stimuli may be required to elicit a cellular response in terms of cell death.

It has been reported that breast tumours express low levels of bax which is associated with resistance to apoptosis (Bargou *et al*., 1995). Overexpression of bax in MCF-7 cells renders them more susceptible to apoptotic stimuli, including irradiation and Fas/Apo-1 induction (Bargou *et al*., 1996; Sakakura *et al*., 1996). Recently, wild type p53 was shown to directly modulate transcription of genes for bax (Miyashita & Reed, 1995) and insulin-like growth factor binding protein-3 (IGFBP-3; Buckbinder *et al*., 1995). Wild type p53 induces apoptosis in a number of cell types (Yonish-Rouach *et al*., 1991; Shaw *et al*., 1992). However, apoptosis may be induced by either p53-dependent or independent pathways in mammary epithelial cells (Merlo *et al*., 1995).

Available data indicates that MCF-7 cells express wild type p53 but that it may be non-functional as it is excluded from the nucleus (Moll *et al*., 1992). We have previously demonstrated the ability of vitamin D derivatives to up-regulate wild type p53 protein expression in MCF-7 cells, which has been shown by others to be translocated to the nucleus where its function is restored (James *et al*., 1995; Elstner *et al*., 1995). Our present results show that treatment of MCF-7 cell by EB1089 led to a modest increase of bax levels over a 6 day period. Whether this observation maybe a consequence of increased expression of wild type p53 by the vitamin D analogue remains to be determined.

Another possible mechanism through which the vitamin D compounds can exert their effects is by disruption of the mitogenic activity of cell survival factors, such as insulin-like growth factor-1 (IGF-1). Studies have shown that EB1089 can diminish the mitogenic activity of IGF-1 in MCF-7 cells (Vink-van Wijngaarden *et al*., 1996; Xie *et al*., 1997) which may occur in part by down-regulation of the IGF-1 receptor (Xie *et al*., 1997). Expression of IGFBP-3 protein is also increased by EB1089 in ER-positive and ER-negative breast cancer cells, which may play a role in sequestering IGF-1, preventing binding to its receptor and suppressing mitogenic activity (Colston *et al*., 1998).

The findings from the present study demonstrate that EB1089 is a potent anti-tumour agent which is capable of inducing apoptosis *in vivo* as well as *in vitro* in breast cancer cells. Whilst the differential regulation of bcl-2 and bax by EB1089 resulted in a lower bcl-2/bax ratio favouring apoptosis, the regulation of other bcl-2 family members and the caspases by the vitamin D analogue will also have to be taken into consideration to fully appreciate the intricate mechanisms involved in apoptosis.

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