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EFFECTS OF SYNTHETIC VITAMIN D ANALOGUES ON BREAST CANCER CELL PROLIFERATION *IN VIVO* AND *IN VITRO*

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Abstract—Calcipotriol (MC903) is a novel vitamin D analogue which effects cellular differentiation and proliferation *in vitro* and has reduced effects on calcium metabolism *in vivo*. In the present study its *in vitro* activity was evaluated using the MCF-7 breast cancer cell line, and its effects on calcium metabolism and mammary tumour growth were measured *in vivo* in adult female rats. Calcipotriol was compared to the natural metabolite of vitamin D_3, 1α,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$] and its synthetic analogue 1α hydroxycholecalciferol [$1\alpha(OH)D_3$]. Both calcipotriol and $1,25(OH)_2D_3$ produced significant inhibition of MCF-7 cell proliferation at a concentration of 5×10^{-11} M. Intraperitoneal administration of calcipotriol to normal female rats showed that the analogue was 100–200 times less active than $1,25(OH)_2D_3$ in raising serum calcium concentration and urinary calcium excretion. Anti-tumour activity of the vitamin D analogues was investigated *in vivo* using the nitrosomethylurea-induced rat mammary tumor model. Rats, maintained on a low calcium diet, were treated with $1\alpha(OH)D_3$ (0.25 and 1.25 μg/kg). Both doses produced a response rate of 25% but hypercalcaemia developed. Treatment with calcipotriol (50 μg/kg) of rats maintained on a normal laboratory diet caused inhibition of tumour progression (response rate 17%) without the development of severe hypercalcaemia. This study supports the concept that vitamin D derivatives may inhibit breast cancer cell proliferation *in vivo*.

The biologically active metabolite of vitamin D, 1α,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$‡], regulates calcium and phosphate transport in the intestine and the mobilization of mineral from bone [1]. Recent evidence suggests that the effects of $1,25(OH)_2D_3$ may be more widespread than formerly appreciated. Receptors for the hormone have been detected not only in the classical target organs, the intestine, kidney and bone, but also in other sites such as the skin [2], pancreas and pituitary [3], and also certain cells of the immune system [4]. A wide variety of human cancer cell lines, including breast, also have this receptor [5, 6]. Other studies have indicated that *in vitro* $1,25(OH)_2D_3$ may influence cellular proliferation [7–9] and differentiation [10–12].

Studies *in vivo* have shown that immunosuppressed mice bearing lung and colon carcinoma and melanoma xenografts appear to regress when they are treated with $1,25(OH)_2D_3$ [13, 14]. Similarly, mice inoculated with M1 myeloid leukaemia cells had a prolonged survival time when treated with the synthetic vitamin D analogue 1α hydroxycholecalciferol [$1\alpha(OH)D_3$] [15] which undergoes 25-hydroxylation in the liver to form $1,25(OH)_2D_3$ and shows the same profile of activity as the native hormone [16]. Our preliminary studies indicate that $1\alpha(OH)D_3$ may inhibit the growth of carcinogen-induced mammary carcinomas [17]. *N*-Methyl-nitrosourea (NMU) induces hormone-dependent mammary tumors in adult female rats and this animal model of breast cancer has been used to study hormonal effects on tumour growth *in vivo* [18].

One major drawback to considering these vitamin D compounds as therapeutic agents in hyperproliferative disorders is that $1,25(OH)_2D_3$ causes hypercalcaemia at doses higher than a few micrograms per day and thus it remains to be established whether analogues of vitamin D can produce significant anti-tumour effects without unacceptable toxicity. Calcipotriol (MC903) is a novel vitamin D analogue which combines potent effects on cell proliferation and differentiation with a decreased activity on calcium metabolism [19] and has been used topically in the treatment of psoriasis [20].

In the present study we have compared effects of $1,25(OH)_2D_3$ and calcipotriol on the proliferation of cultured breast cancer cell lines. Furthermore, we have compared the effects of calcipotriol and $1\alpha(OH)D_3$ on both calcium metabolism and the progression of NMU-induced rat mammary tumours. In order to probe the mechanism by which vitamin D analogues might influence the growth of these oestrogen-responsive tumours we have assessed the binding of vitamin D derivatives to the oestrogen receptor *in vitro* and interactions between $1\alpha(OH)D_3$ and 17β oestradiol *in vivo*.

MATERIALS AND METHODS

Compounds. Crystalline $1,25(OH)_2D_3$ was a

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‡ Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxycholecalciferol; $1\alpha(OH)D_3$, 1α hydroxycholecalciferol; NMU, *N*-methyl-nitrosourea; VDR, vitamin D receptor; ER, oestrogen receptor.

generous gift from Dr W. Meier, Hoffmann-La Roche and Co. (Basel, Switzerland). 1α(OH)D_3 and calcipotriol (MC903) were gifts from Dr L. Binderup, Leo Pharmaceuticals (Denmark). For the *in vitro* studies compounds were dissolved in absolute ethanol. For the *in vivo* studies compounds were dissolved in steroid suspension medium (0.9% sodium chloride, 0.5% sodium carboxy-methyl cellulose, 0.4% Tween 80 and 0.9% benzyl alcohol) or propylene glycol. 1α,25-Dihydroxy-[26,27-*methyl*-^{3}H]cholecalciferol (180 Ci/mmol), 25-hydroxy-[23,24,-*N*-^{3}H]cholecalciferol (180 Ci/mmol), [2,4,6,7-^{3}H]oestradiol (100 Ci/mmol) and [^{3}H]-thymidine (5 Ci/mmol) were obtained from Amersham International (Amersham, U.K.). Other radioinert chemicals were purchased from Sigma Chemical Co. (Poole, U.K.). Tissue culture medium and reagents were obtained from Gibco (Paisley, U.K.).

Cellular effects in vitro. MCF-7 human breast cancer cells were grown in Dulbecco's modification of Eagle's minimal essential medium 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 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 calcipotriol. After incubating at 4° for 18 hr, the bound and free sterols were separated by the hydroxylapatite method as described previously [21]. Binding of vitamin D derivatives to serum binding proteins was studied using rat serum diluted 1:8000 in 0.05 M phosphate buffer (pH 7.4 containing 0.01% human γ globulin). Aliquots (400 μL) were incubated at 4° for 2 hr with 10,000 dpm of [^{3}H]-25$(OH)D_3$ with or without increasing concentrations of radioinert 25$(OH)D_3$, 1α$(OH)D_3$, 1,25$(OH)_2D_3$ or calcipotriol. After incubating at 4° for 2 hr, the bound and free sterols were separated by the addition of dextran-coated charcoal. Radioactivity was assessed by liquid scintillation counting.

For studies on cell proliferation, MCF-7 cells were resuspended at 1×10^4 cells/mL in RPMI 1640 medium containing 2.5% charcoal-stripped foetal calf serum, 2 mM glutamine penicillin (100 U/mL) and streptomycin (100 mg/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 an incubator with a humidified 5% CO_2 atmosphere. Medium was removed and fresh medium containing calcipotriol or 1,25$(OH)_2D_3$ (10^{-8}–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 [22] with six replicate cultures for each concentration tested.

Effects on calcium metabolism. Female Wistar rats (200–250 g) were treated with 1,25$(OH)_2D_3$ (0.5 μg/kg), 1α$(OH)D_3$ (1 μg/kg) or calcipotriol (5 and 100 μg/kg) intraperitoneally. Compounds were given on alternate days for 2 weeks in steroid suspension medium [1α$(OH)D_3$] or propylene glycol [1,25$(OH)_2D_3$ and calcipotriol]. Each treatment group consisted of four to six rats. Control rats received the appropriate vehicle and standard laboratory diet containing 1% calcium and 0.75% phosphorous. Twenty-four hours prior to the end of the treatment period the rats were placed in individual metabolic cages and urine was collected. At the end of the experiment, animals were exsanguinated by cardiac puncture under halothane anaesthesia. Serum was stored at −20° until analysed.

Treatment of tumour-bearing rats. An inbred strain of virgin female Ludwig/Wistar/Olac rats bearing mammary tumours induced by NMU (Harlan OLAC Ltd, U.K.) were kept as described previously [18].

In studies to determine the response of NMU-induced tumours to 1α$(OH)D_3$ given intraperitoneally, all rats were maintained on a low calcium diet (0.1% calcium diet obtained from Labsure, Cambridge, U.K.) for 1 week prior to start of treatment of hypercalcaemia. In each study rats bearing at least one assessable tumour (>15 mm in diameter) were randomly assigned to treated or control groups. Rats were injected thrice weekly with steroid suspension medium with or without vitamin D analogues. Treatment was generally continued for 28 days and tumour volume was determined weekly. In all experiments tumour volume was determined by measuring the two largest diameters at right angles, using vernier callipers. 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 week 0 (100%) was calculated for each rat. Animals whose tumours showed signs of ulceration or in which tumour burden became excessive (>10% body weight) were killed by terminal anaesthesia. At the end of each experiment, surviving animals were exsanguinated under halothane anaesthesia. Tumours were excised, weighed and immediately frozen in liquid nitrogen and stored at −70°. Serum was stored at −20° until analysed.

In the first study 24 rats were treated with 1α$(OH)D_3$ (0.5 μg/kg) or vehicle control. In a second study with a higher dose (1.25 μg/kg), 36 tumour-bearing rats were randomly assigned to one of three groups. The first received 1α$(OH)D_3$ (1.25 μg/kg) alone, the second received vehicle (steroid suspension medium) alone and, to investigate the effect of 1α$(OH)D_3$ on oestrogen-stimulated

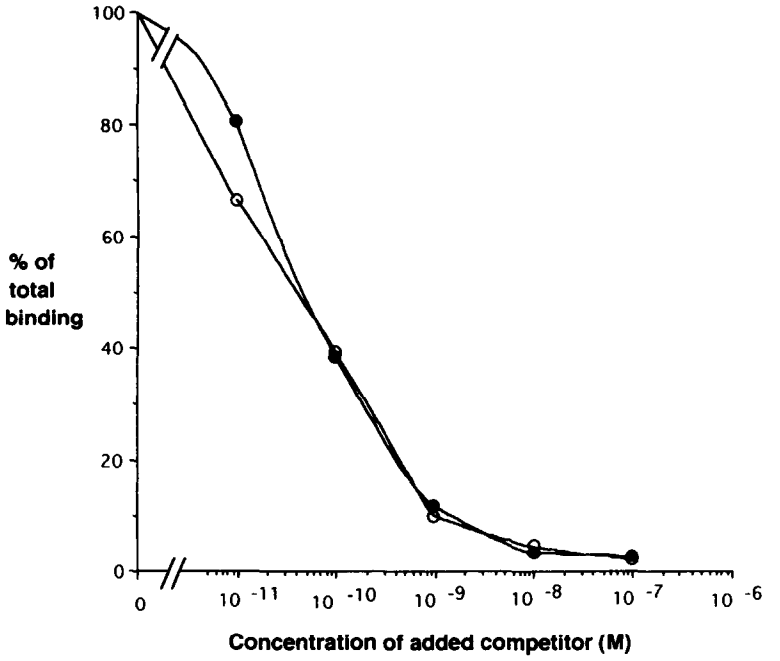

Fig. 1. Receptor binding of $1,25(OH)_2D_3$, calcipotriol and $1\alpha(OH)D_3$. KTEDM cytosol from MCF7 cells was labelled as described in the text with 1.3 nM $[^3H]1,25(OH)_2D_3$ in the presence of increasing concentrations of unlabelled $1,25(OH)_2D_3$ (○) and calcipotriol (●). Displacement of $[^3H]1,25(OH)_2D_3$ is shown as % of radioactivity bound in the absence of added compounds. Results are representative of three separate experiments.

mammary tumour growth, a third group received $1\alpha(OH)D_3$ (1.25 μg/kg) plus 17β oestradiol (1 μg/kg body weight) subcutaneously thrice weekly. Treatment was continued for up to 41 days in this experiment.

In a third study 24 rats were treated with calcipotriol (50 μg/kg) or control (vehicle alone). Rats were maintained on a normal laboratory diet in this experiment.

Other methods. Serum calcium, phosphate, albumin and creatinine were measured on a Technicon RA-1000 analyser. To determine VDR content of tumours, tumour tissue was thawed on ice and KTEDM extracts were assayed for unoccupied VDR as described previously [21]. Tumour cytosols containing high levels of oestrogen receptor (ER) were labelled with $[^3H]17\beta$ oestradiol as described previously [23].

Statistical methods. The animals were categorized into three groups based on the change in tumour volume between 0 and 28 days: (A) those with 50% or greater reduction in total volume, response; (B) those with a reduction in total volume less than 50%, regression; (C) those with no change or an increase in total tumour volume, progression. Those animals that died before the termination of the experiment were allocated to group C. Percentage change in total tumour volume at each week of each study was compared between groups using the non-parametric Mann–Whitney *U*-test.

Due to the non-normality of the data, comparisons of the biochemical parameters also used the non-parametric Mann–Whitney *U*-test.

Comparisons of *in vitro* cell proliferation results were made using the Student's *t*-test.

RESULTS

In vitro *effects of vitamin D derivatives*

The ability of synthetic vitamin D analogues to bind to VDR in breast cancer cells (MCF-7) was examined.

Figure 1 shows that half-maximal displacement of bound $[^3H]1,25(OH)_2D_3$ was obtained with calcipotriol at a concentration of approximately 7×10^{-11} M and with $1,25(OH)_2D_3$ at a concentration of approximately 6×10^{-11} M. A much higher concentration (2.7×10^{-7} M) of $1\alpha(OH)D_3$ was required to achieve a half-maximal displacement (data not shown).

To compare the abilities of $1\alpha(OH)D_3$ and calcipotriol to bind to transport proteins in rat serum, competition studies were carried out. Figure 2 shows that, as expected, $1,25(OH)_2D_3$ was a less potent competitor for $[^3H]25(OH)D_3$ binding than $25(OH)D_3$ itself. Displacement curves for $1\alpha(OH)D_3$ and calcipotriol were similar, demonstrating that the affinity for these compounds is approximately 1000-fold lower than for $25(OH)D_3$.

In order to investigate whether vitamin D analogues might bind to oestrogen receptors in tumour tissue, competition studies were carried out. Figure 3 demonstrates that neither $1,25(OH)_2D_3$ nor calcipotriol were able to compete for specific $[^3H]$-oestradiol binding sites in tumour tissue but that, as expected, diethylstibestrol was a potent competitor.

In order to compare effects of $1,25(OH)_2D_3$ and calcipotriol on the proliferation of MCF-7 cells, cultures were incubated for 4–10 days in the presence or absence of the vitamin D derivatives. Significant inhibition of $[^3H]$thymidine incorporation was seen

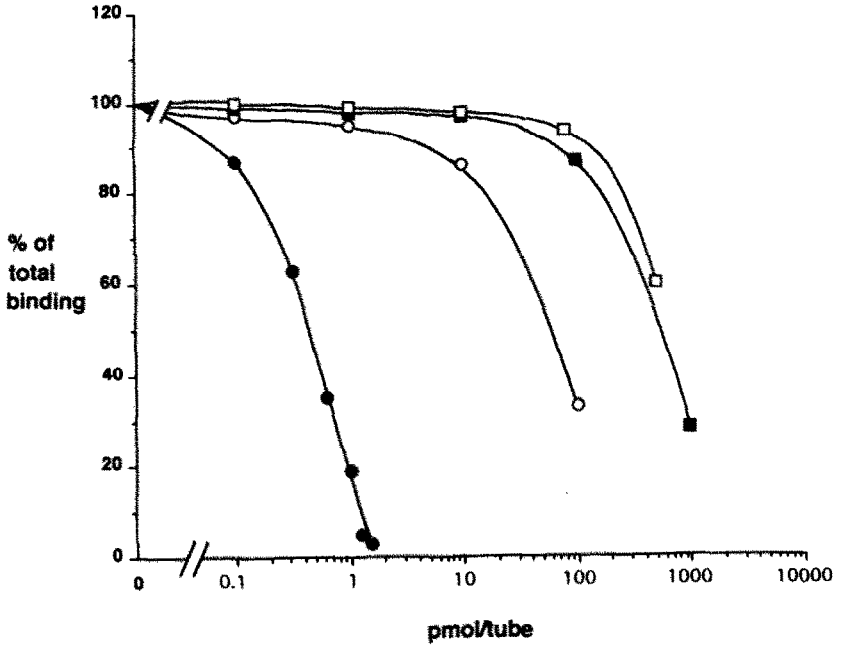

Fig. 2. Binding of vitamin D derivatives to serum binding proteins. The displacement of [^{3}H]25(OH)D_3 from rat serum by increasing concentrations of radioinert 25(OH)D_3 (●), 1,25$(OH)_2D_3$ (○) calcipotriol (■) and 1α(OH)D_3 (□) is expressed as % of radioactivity bound in the absence of added compounds. Results are representative of three separate experiments.

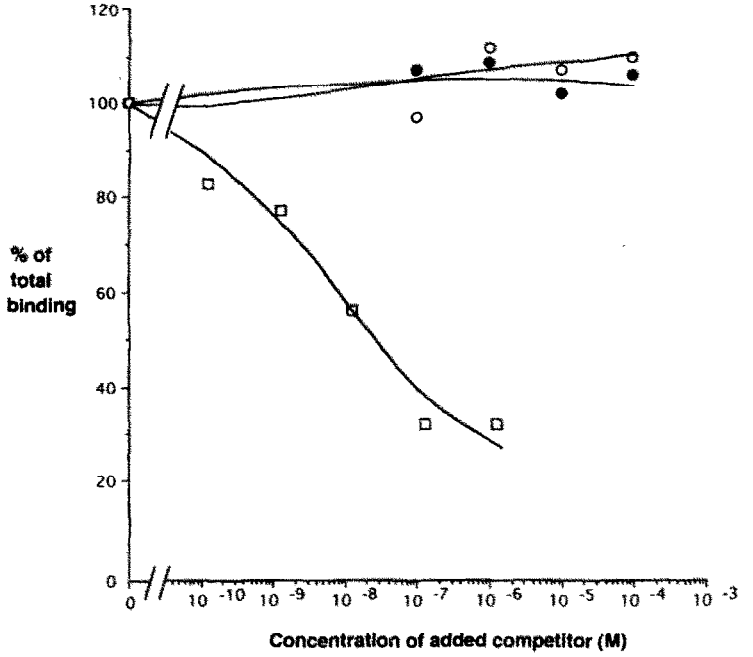

Fig. 3. Displacement of [^{3}H]oestradiol from ER in MCF-7 cell cytosol by diethylstilbestrol (□), 1,25$(OH)_2D_3$ (●) and calcipotriol (○). Results are expressed as the percentage of radioactivity bound in the presence of varying amounts of competitor and are representative of two separate experiments.

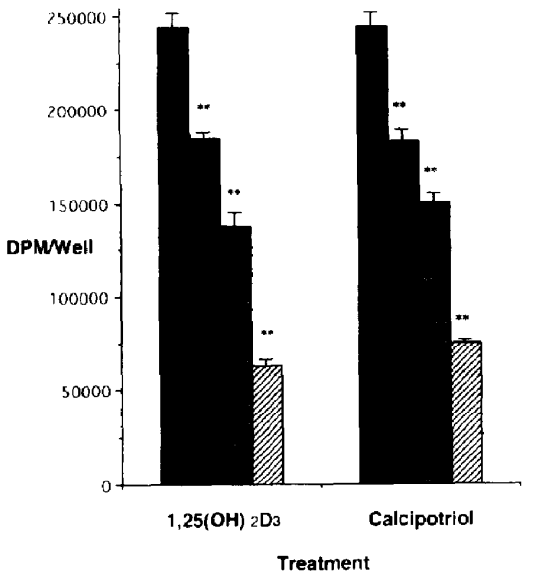

Fig. 4. [^{3}H]Thymidine incorporation in MCF-7 cells in response to treatment with $1,25(OH)_2D_3$ or calcipotriol. Results show means ± SD of six replicates and are after 10 days of treatment. $^{**}P < 0.01$ (■) Control, (■) 5×10^{-11}, (■) 5×10^{-10} M, (▨) 5×10^{-9} M.

with both compounds. Figure 4 demonstrates that calcipotriol was approximately equipotent with $1,25(OH)_2D_3$. Both compounds produced greater than 70% inhibition of [^{3}H]thymidine incorporation at a concentration of 5×10^{-9} M after 10 days. Effects on cell proliferation were also examined by assessing numbers of viable cells in control and $1,25(OH)_2D_3$ treated cultures by Trypan blue dye exclusion. A dose-dependent increase in cell number was observed with $1,25(OH)_2D_3$ which correlated with the inhibition of [^{3}H]thymidine incorporation. Similar results were obtained with ZR-75-1 breast cancer cells (data not shown).

Effects on calcium metabolism

Serum calcium concentration (Fig. 5A) and urinary calcium excretion (Fig. 5B) were compared after treatment with $1,25(OH)_2D_3$, $1\alpha(OH)D_3$ and calcipotriol in normal animals. Treatment with both $1,25(OH)_2D_3$ (0.5 μg/kg/day) and $1\alpha(OH)D_3$ (1.0 μg/kg/day) produced significant increases in serum calcium concentration and urinary calcium excretion. However, calcipotriol at a dose of 5 μg/kg did not significantly raise serum calcium although urinary calcium excretion was moderately increased. A dose of 100 μg/kg was required to produce significant hypercalciuria and hyperlcalcaemia. The results show that the systemic effects of calcipotriol on calcium metabolism as assessed by the elevation of serum calcium concentration and increased urinary calcium excretion are seen at doses 100–200 times greater than those of $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$.

Effects of vitamin D analogues on rat mammary tumour progression

The *in vivo* effects of vitamin D analogues on rat mammary tumour progression are summarized below. The effect of 0.5 μg/kg $1\alpha(OH)D_3$ on rat mammary tumour progression is shown in Fig. 6 and Table 1(a). At this dose $1\alpha(OH)D_3$ significantly inhibited tumour progression when compared with the control group ($P = 0.03$). All animals receiving this dose of $1\alpha(OH)D_3$ survived the treatment period and one control animal was killed because of excessive tumour burden. This treatment regimen produced a significant elevation in serum calcium (Table 1) and phosphate concentrations ($P \leq 0.001$).

The effect of treatment with 1.25 μg/kg $1\alpha(OH)D_3$ on rat mammary tumour progression is given in Table 1(b). Percentage change in tumour volume was smaller in treated rats than in control animals, although the difference was not statistically significant at 27 days of treatment ($P = 0.86$). In rats treated with 17β oestradiol together with $1\alpha(OH)D_3$, no increase in tumour volume was observed ($P = 0.29$). There was a significant elevation of serum calcium in the two groups of rats receiving the higher dose of $1\alpha(OH)D_3$ alone and in combination with 17β oestradiol (Table 1).

The effects of calcipotriol at a dose of 50 μg/kg on rat mammary tumour progression are shown in Table 1(c) and in Fig. 7. Calcipotriol appeared to inhibit tumour growth although the results are only marginally significant after 28 days of treatment ($P = 0.07$). There was a small but significant elevation in serum calcium with calcipotriol treatment compared with controls ($P < 0.02$, Table 1). No significant decrease in overall body weight was observed after 4 weeks of treatment with the vitamin D analogues (data not shown).

Expression of VDR in NMU-induced rat mammary tumours

All NMU-induced mammary tumours from a range of untreated rats contained specific [^{3}H]-$1,25(OH)_2D_3$ binding activity. Receptor content ranged from 16–133 fmol/mg of cytosol protein (median 44 fmol/mg). Tumour VDR content was also measured in those rats treated with 0.5 μg/kg $1\alpha(OH)D_3$ for 4 weeks. The VDR content of these tumours ranged from 5 to 149 fmol/mg (median = 21 fmol/mg). The values were not significantly different ($P = 0.10$).

DISCUSSION

There is now some evidence that the active hormonal form of vitamin D, $1,25(OH)_2D_3$, can inhibit proliferation of a number of established human cancer cell lines including breast cancer, melanoma, colon cancer and leukaemic cells and can stimulate differentiation in other cell types [9–13, 24–27]. Less is known of the antitumour effects of $1,25(OH)_2D_3$ *in vivo*. Both $1,25(OH)_2D_3$ and the synthetic analogue $1\alpha(OH)D_3$ have been shown to prolong the survival time of mice inoculated with leukaemia cells [15]. Inhibitory effects of $1,25(OH)_2D_3$ on mouse skin tumour development have been reported [28]. In contrast, Yamaoka *et al.* [29] presented evidence that growth of a VDR-positive osteosarcoma cell line inoculated into congenitally athymic mice was stimulated by

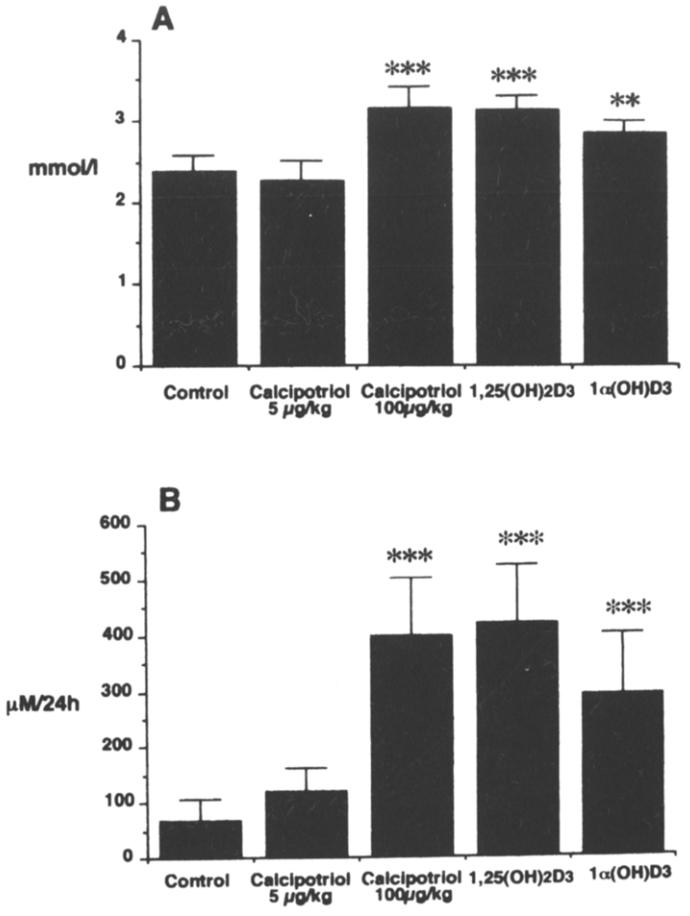

Fig. 5. Effects of $1,25(OH)_2D_3$, $1\alpha(OH)D_3$ and calcipotriol on calcium metabolism in normal rats. (A) Effects on serum calcium (mmol/L). (B) Effects on urinary calcium excretion (mmol/24 hr). In both cases graphs show means ± SD from 4–6 animals. $^{**}P < 0.01$, $^{***}P < 0.005$.

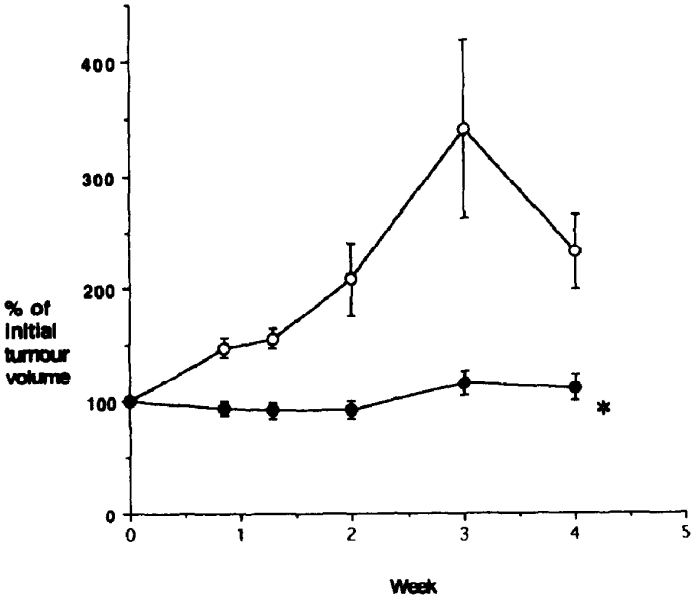

Fig. 6. Effects of $1\alpha(OH)D_3$ (0.5 μg/kg) (●) or vehicle control (○) on rat mammary tumour growth. Weekly changes in tumour volume relative to week 0 are expressed as means ± SEM with 12 animals/group. $^{*}P < 0.05$.

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

Treatment	Number of rats	Number of tumours	Regression		Progression	New tumours	Response rate (%)*	P value	Number of deaths		Serum calcium (mmol/L)‡
			>50%	<50%					Tumour burden	Presumed hypercalcaemia	
(a) $1\alpha(OH)D_3$ (0.5 μg/kg)											
Control	12	12	0	1	11	0	0		1	0	2.53 (2.48–2.78)
$1\alpha(OH)D_3$	12	12	1	3	8	0	25	0.03	0	0	2.72§ (2.57–3.50)
(b) $1\alpha(OH)D_3$ (1.25 μg/kg)											
Control	12	13	0	1	11	2	0		1 (4)†	0 (0)	2.48 (2.37–2.72)
$1\alpha(OH)D_3$	12	12	3	1	8	0	25	0.86	0 (1)	3 (0)	2.85§ (2.56–3.22)
$1\alpha(OH)D_3$ + 17β oestradiol	12	12	3	2	7	0	25	0.29	2 (1)	0 (0)	2.95§ (2.58–3.50)
(c) Calcipotriol (50 μg/kg)											
Control	12	14	0	2	10	4	0		0	0	2.65 (2.46–2.79)
Calcipotriol	12	13	2	5	5	0	17	0.07	0	0	2.76‖ (2.60–2.80)

Rats treated with $1\alpha(OH)D_3$ were maintained on a low calcium diet as described in Materials and Methods. Rats treated with calcipotriol received normal laboratory diet.

* Response rate (%) $= \frac{\text{Number of animals with greater than 50\% regression} \times 100}{\text{Total number of animals}}$.

† Numbers in parentheses show deaths between 27 and 41 days.

‡ Serum calcium was measured on days 28 (a and c) and 41 (b).

§$P < 0.001$, ‖$P < 0.02$.

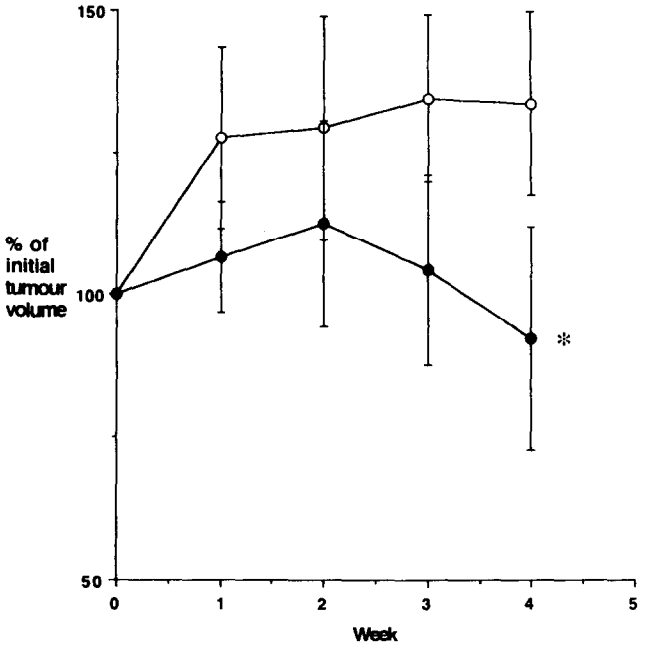

Fig. 7. Effects of calcipotriol (50 μg/kg) (●) or vehicle control (○) on rat mammary tumour growth. Weekly changes in tumour volume relative to week 0 are expressed as means ± SEM with 12 animals/group. *P = 0.07.

$1,25(OH)_2D_3$. Other studies in immunosuppressed mice with solid tumour xenografts developed from VDR-positive human colon and melanoma cell lines showed inhibition of tumour growth which was not seen with xenografts of a receptor negative cell line [14]. However, in this study animals were maintained on a low calcium diet to prevent hypercalcaemia. The major drawback to considering conventional vitamin D compounds as therapeutic agents in hyperproliferative disorders is their potent effects on calcium metabolism, since $1,25(OH)_2D_3$ causes hypercalcaemia at doses higher than a few micrograms per day.

These considerations have prompted interest in the development of novel vitamin D analogues designed to separate the hypercalcaemic from growth-inhibitory effects displayed by $1,25(OH)_2D_3$. Among such analogues, calcipotriol has been shown to have direct effects on cell proliferation and differentiation coupled with a decreased activity on calcium mobilization [19, 30]. Our findings on the relative potency of calcipotriol, $1,25(OH)_2D_3$ and $1\alpha(OH)D_3$ on serum calcium concentrations and urinary calcium excretion in rats are very similar to those reported previously [30, 31], with calcipotriol exhibiting only 0.5–1% of the activity of the two reference compounds. Furthermore, our studies on the binding of these compounds to rat serum transport proteins indicate that calcipotriol and $1\alpha(OH)D_3$ display similar binding characteristics while calcipotriol possesses a much lower activity in mobilizing calcium, indicating that its lower potency is unlikely to be solely attributable to differences in serum transport and delivery to target organs.

Calcipotriol has been reported to be of comparable potency with $1,25(OH)_2D_3$ in inducing differentiation and inhibiting proliferation of the human histiocytic lymphoma cell line U937 [19, 26], and also in stimulating osteoclast recruitment [32]. Our results with the MCF-7 breast cancer cell line demonstrate that calcipotriol also has a similar potency to $1,25(OH)_2D_3$ in these cells. The inhibitory effects of $1,25(OH)_2D_3$ on cell proliferation are thought to be receptor mediated, and our competition studies have demonstrated that calcipotriol appears to bind to VDR in human cancer cells in a manner similar to $1,25(OH)_2D_3$. The related analogue $1\alpha(OH)D_3$ was more than 1000 times less efficient than radioinert $1,25(OH)_2D_3$ and calcipotriol in displacing $[^3H]$-$1,25(OH)_2D_3$ from binding to VDR, since $1\alpha(OH)D_3$ requires conversion to $1,25(OH)_2D_3$ before binding to the receptor.

There are few reported studies regarding the effects of $1,25(OH)_2D_3$ *in vivo* on mammary tumours. Using the 7.12 dimethylbenzanthracene-induced rat mammary tumour model, Noguchi *et al.* [33] reported that topical administration of $1,25(OH)_2D_3$ had no significant effect on tumour size or incidence. However, these authors did not demonstrate that such tumours contain $1,25(OH)_2D_3$ receptors and are thus potentially responsive to possible growth-inhibiting effects of the steroid. We have assessed the effects of $1\alpha(OH)D_3$ and calcipotriol *in vivo* on the progression of NMU-induced rat mammary tumours. This model has proved useful in the study of hormonal effects on breast tumour growth *in vivo* [18] and we have shown previously that these tumours contain VDR [21]. Our results demonstrate that $1\alpha(OH)D_3$ both inhibits the progression of these mammary tumours and prevents the stimulatory effects of 17βoestradiol. The doses of this synthetic vitamin D analogue used

in our study were similar to those adopted by Honma *et al.* [15] to treat nude mice inoculated with M1 myeloid leukaemia cells but were more than 10-fold greater than the usual therapeutic doses of $1\alpha(OH)D_3$. The effective dose range is narrow, however, with the higher dose causing an unacceptable degree of hypercalcaemia despite the maintenance of animals on a low calcium diet. Experiments with cultured breast cancer cells have suggested that an increase in extracellular calcium concentration antagonizes the growth inhibitory effect of $1,25(OH)_2D_3$ [34]. This observation may explain in part why the higher dose of $1\alpha(OH)D_3$ used in our study (and which was associated with increased mortality due to hypercalcaemia) was less effective in inhibiting tumour progression.

In view of the potency of calcipotriol in inhibiting breast cancer cell growth *in vitro* coupled with its less potent effects on calcium homeostasis we examined the effect of this analogue at a dose of 50 μg/kg on the progression of NMU-induced mammary tumours. This dose regimen did not produce a highly significant elevation of serum calcium, and the tumour growth of animals in the treated group was inhibited with a regression of 58% compared with only 17% in controls. Recent evidence has indicated that the metabolic half-life of calcipotriol *in vivo* is less than 10 min [31] and this is likely to contribute to its decreased potency compared with $1,25(OH)_2D_3$ on calcium mobilization. In view of this, it is surprising that in our study effects of the vitamin D analogue on tumour progression were seen, indicating that the compound appears to possess anti-tumour activity despite its rapid metabolic clearance *in vivo*. It may be that the exposure of neoplastic cells to such compounds for only a short time is sufficient to initiate intracellular events leading to the inhibition of cell replication, whereas the continued presence of vitamin D compounds is required for the "classical" vitamin D effects on calcium homeostasis to be produced.

The mechanism by which $1,25(OH)_2D_3$ and vitamin D analogues may modulate breast tumour growth *in vivo* is not yet clear. It is well documented that NMU-induced mammary tumours are oestrogen dependent. Tumours regress when rats are ovariectomized while oestradiol treatment accelerates tumour growth [18]. Topical administration of $1,25(OH)_2D_3$ to rats bearing 7.12 dimethylbenzanthracene-induced mammary tumours was associated with suppression of ovarian function and a reduction of tumour ER content [33]. However, in this study no significant effect of $1,25(OH)_2D_3$ on tumour growth or development was seen. $1\alpha(OH)D_3$ does not appear to be exerting its effects indirectly through suppression of ovarian function or tumour responsiveness to oestrogen, since serum 17β oestradiol concentrations and tumour oestrogen receptor content are not significantly altered in rats treated with the analogue (our unpublished observations). Vitamin D analogues were also unable to bind to oestrogen receptors from tumour tissue *in vitro*. Possible mechanisms may involve alteration in oncogene expression since $1,25(OH)_2D_3$ has been shown to down-regulate transcription of the c-*myc* proto-oncogene in HL-60 promyelocytic leukaemia cells [35, 36]. Alterations in secretion or responsiveness to growth factors may also be involved [37–40]. However, our results suggest that effects of vitamin D analogues on tumour growth are mediated through the $1,25(OH)_2D_3$ receptor, since calcipotriol and $1,25(OH)_2D_3$ have similar properties in MCF7 cells.

In a recent study of 14 patients with locally advanced or cutaneous metastatic breast cancer treated topically for 6 weeks with calcipotriol, three had a partial response and one a minimal response of the treated lesion. All patients who responded to treatment had receptor-positive tumours [41].

Taken together these results indicate that analogues of vitamin D which promote differentiation and inhibit cancer cell growth but which have reduced calcaemic activity may have clinical potential as anti-tumour agents. The development of such compounds may lead to additional therapeutic strategies for the control of breast cancer and other proliferative diseases.

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