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Inhibition of Breast Cancer Cell Growth by Combined Treatment with Vitamin D₃ Analogues and Tamoxifen

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ABSTRACT

The steroid hormone 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] has potential to be used as an antitumor agent, but its clinical application is restricted by the strong calcemic activity. Therefore, new vitamin D₃ analogues are developed with increased growth inhibitory and reduced calcemic activity. In the present study, we have examined the antiproliferative effects of four novel vitamin D₃ analogues (CB966, EB1089, KH1060, and 22-oxa-calcitriol) on breast cancer cells, either alone or in combination with the antiestrogen tamoxifen. The estrogen-dependent ZR-75-1 and estrogen-responsive MCF-7 cell lines were used as a model. It was shown that, with EB1089 and KH1060, the same growth inhibitory effect as 1,25-(OH)₂D₃ could be reached at up to 100-fold lower concentrations, whereas CB966 and 22-oxa-calcitriol were nearly equipotent with 1,25-(OH)₂D₃. The growth inhibition by the vitamin D₃ compounds could be augmented by combined treatment with tamoxifen. At the maximal effective concentrations of the vitamin D₃ compounds, the effect of combined treatment was additive (MCF-7 cells) or less than additive (ZR-75-1 cells). Tamoxifen increased the sensitivity of the cells to the vitamin D₃ compounds 2- to 4000-fold, which was expressed by a shift to lower median effective concentration values. Thereby, the vitamin D₃ compounds may be used at even lower dosages in combination therapy with tamoxifen. A major problem of tamoxifen therapy is the development of tamoxifen resistance. We have observed that tamoxifen-resistant clones of ZR-75-1 cells retain their response to the vitamin D₃ compounds. Regulation of the growth-related oncogene c-*myc* (mRNA level) and the estrogen receptor (protein level) were studied but appeared not to be related to the antiproliferative action of the vitamin D₃ compounds. Together, our data point to a potential benefit of combination therapy with 1,25-(OH)₂D₃ or vitamin D₃ analogues and tamoxifen for the treatment of breast cancer.

INTRODUCTION

The seco-steroid hormone 1,25-(OH)₂D₃,[2] the biologically most active metabolite of vitamin D₃, is a well-known regulator of calcium homeostasis and bone metabolism (1). Over the last decade, evidence has accumulated that 1,25-(OH)₂D₃ is also involved in the regulation of proliferation and differentiation of cells and tissues not primarily related to mineral metabolism (2). Also, in breast cancer cells and tumors, an antiproliferative effect of 1,25-(OH)₂D₃ has been demonstrated (3–5). These nonclassical effects offer promise for the use of 1,25-(OH)₂D₃ as an antiproliferative drug (6). A major drawback for its clinical application is that high doses are needed for tumor suppression, which may result in negative side effects like the development of hypercalcemia. Therefore, vitamin D₃ analogues were developed in an attempt to dissociate effects on growth and differentiation from effects on intestinal calcium absorption and bone resorption.

Most analogues synthesized have modifications in the side-chain of the 1,25-(OH)₂D₃ molecule. The vitamin D₃ side-chain analogues used in this study, CB966, EB1089, KH1060, and OCT, have been shown to be more potent than 1,25-(OH)₂D₃ in the inhibition of proliferation of leukemic cells, whereas the calcemic activity *in vivo* was similar or even weaker (7–10). A few studies have also demonstrated antiproliferative effects of OCT, EB1089, and KH1060 on breast cancer cells in culture (8, 11–13). In addition, in animal models for breast cancer, OCT and EB1089 suppressed tumor growth without development of hypercalcemia (11, 12). Therefore, these data, together with the high incidence of vitamin D receptors in human breast tumors (14, 15), demonstrate the potential role of vitamin D₃ analogues in the treatment of breast cancer. Presently, the mechanism of the suppression of tumor growth by 1,25-(OH)₂D₃ and analogues is still unclear.

The growth of normal and many breast carcinoma cells is regulated by estrogens. Antiestrogens are effective in controlling the growth of estrogen-responsive tumors, and the antiestrogen tamoxifen is currently widely used in endocrine therapy for breast cancer (16). During prolonged treatment, however, most tumors become eventually resistant to tamoxifen (17). For ER-negative tumors and tamoxifen-resistant tumors, therapeutic choices are limited. Because 1,25-(OH)₂D₃ and analogues have been shown to inhibit breast cancer growth irrespective of the estrogen dependence (4, 11), treatment with vitamin D₃ analogues may be effective for ER-positive, ER-negative, and tamoxifen-resistant breast tumors. Moreover, combined treatment with tamoxifen and vitamin D₃ analogues may provide a more beneficial effect on breast cancer.

In an earlier report, we established a complementary action of tamoxifen and 1,25-(OH)₂D₃ on the growth of MCF-7 and ZR-75-1 estrogen-responsive breast cancer cells (18). In view of the promising effects of low calcemic vitamin D₃ analogues (7–9, 11–13), we examined in the present study whether these compounds, in combination with tamoxifen, resulted in an even better inhibition of breast cancer cell growth. In addition, we have studied ER regulation and evaluated a possible role of the growth-related oncogene c-*myc* in the growth inhibition by 1,25-(OH)₂D₃ and analogues.

MATERIALS AND METHODS

Chemicals. 1,25-(OH)₂D₃, CB966, EB1089, and KH1060 were kindly donated by Dr. L. Binderup of LEO Pharmaceutical Products (Ballerup, Denmark) and OCT by Dr. N. Kubodera of Chugai Pharmaceutical Co., Ltd. (Shizuoka, Japan). The chemical structures of the vitamin D₃ compounds are depicted in Fig. 1. [23,24-³H]1,25-(OH)₂D₃ (120 Ci/mmol) was purchased from Amersham International (Aylesbury, United Kingdom). 17β-E₂, tamoxifen, RPMI 1640, human transferrin, and sodium selenite were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin fraction V was from ICN Biomedicals, Inc. (Costa Mesa, CA). Glutamine, penicillin, streptomycin, and FCS were obtained from Life Technologies (Breda, the Netherlands). Insulin (Actrapid) was from Novo Nordisk A/S (Bagsvaerd, Denmark).

Cell Culture. MCF-7 and ZR-75-1 cells were generously provided by Dr. J. A. Foekens (Department of Endocrine Oncology, Dr. Daniel den Hoed Cancer Center). MCF-7 cells were maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 24 mM

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Fig. 1. Chemical structures of 1,25-(OH)₂D₃ and vitamin D₃ side-chain analogues. The analogues have different types of side-chain modifications: elongation (CB966, EB1089, and KH1060), introduction of an oxygen atom at the C22 position (OCT and KH1060), unsaturation (EB1089), and altered conformation at the C20 position (KH1060).
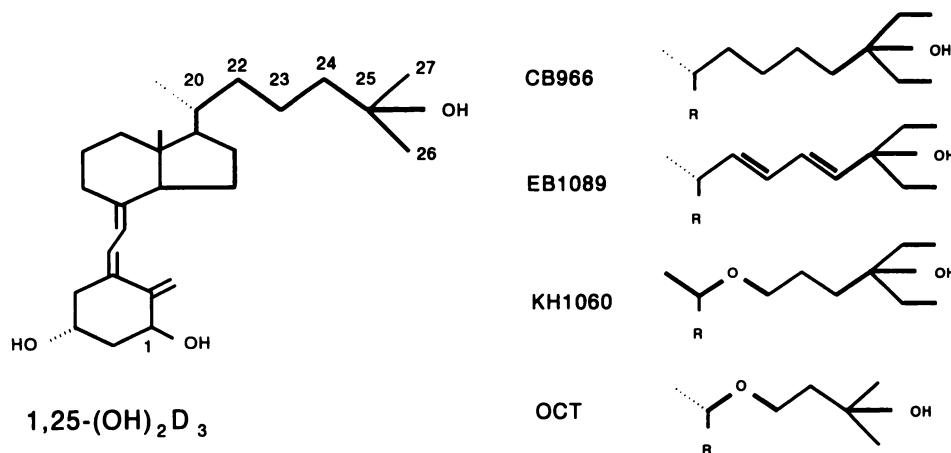
sodium bicarbonate (basal RPMI medium), 10% FCS, and 10 μg/ml insulin. ZR-75-1 cells were maintained in basal RPMI medium supplemented with 10% FCS and 1 nM 17β-E₂. Both cell lines were passaged weekly. Tamoxifen-resistant derivatives of ZR-75-1 cells were isolated and cultured as described previously (19). ZR/HERc cells, which are EGF receptor-positive derivatives of ZR-75-1 cells, were isolated and cultured as described by Van Agthoven et al. (20).

Growth Experiments. Cells were seeded into 6-well dishes at a density of 16,000 cells/cm² for MCF-7 and 32,000 cells/cm² for ZR-75-1 cells in phenol red-free basal RPMI medium supplemented with 2% charcoal-treated FCS. Cells were allowed to attach for 24 h. Next, medium was changed to SFM (basal RPMI medium supplemented with 30 nM sodium selenite, 10 μg/ml transferrin, and 0.2% bovine serum albumin). After another 24 h, the medium was refreshed, and the agents to be tested or vehicle (0.2% ethanol) was added. Medium and test agents were replaced after 2 and 5 days of incubation, and after 8 days of incubation, DNA content was measured using the ethidium bromide method as described previously (18). Proliferation experiments with tamoxifen-resistant ZR-75-1-derived cells and ZR/HERc cells were performed using crystal violet absorbance (21). Parental ZR-75-1 cells were seeded 2000 cells/well; clones XI 13 and VIII 24 (19), 5000 cells/well; and ZR/Herc cells (20), 2200 cells/well into 96-well microplates in basal RPMI medium supplemented with 10% FCS. 10⁻¹⁰ M 17β-E₂ was added to parental ZR-75-1 cells and 10 ng/ml EGF to ZR/Herc cells. Cells were incubated 5 to 7 days after a single addition of the vitamin D₃ compounds.

RNA Isolation and Hybridization. To study the effects of 17β-E₂ and vitamin D₃ compounds on c-myc mRNA expression we have used a similar incubation procedure as in the growth experiments, i.e., 24 h after seeding (1.5 × 10⁶ cells in 25 cm² culture flasks) in basal RPMI medium with 2% charcoal-treated FCS; medium was changed to SFM, and after another 24 h, test agents were added. Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (22). Samples of 10–20 μg RNA were fractionated on a 1% agarose-2% formaldehyde gel and transferred onto a Hybond N+ nylon membrane (Amersham; Ref. 23). The membrane was prehybridized for at least 2 h at 42°C in hybridization buffer [50% formamide, 6% dextran sulfate, 1 × Denhardt's solution, 1 μg/ml denatured herring sperm DNA, 5X SSC (1X SSC = 150 mM sodium chloride-15 mM sodium citrate), 0.2% SDS, and 0.02 M NaH₂PO₄]. Hybridization was carried out at 42°C for 16–24 h. The probes, a 1.4-kilobase ClaI-EcoRI fragment of the human c-myc gene (24) and a 0.8-kilobase EcoRI-HindIII fragment of the human GAPDH gene (25), were labeled with [³²P]ATP using random primers. After hybridization, membranes were washed twice in 2X SSC-0.1% SDS for 5 min at room temperature, twice in 2X SSC-0.1% SDS for 20 min at 42°C, and twice in 0.5X SSC-0.1% SDS for 20 min at 42°C. Membranes were exposed to medical X-ray films (Fuji Photo Film Co., Tokyo, Japan), and autoradiographs were quantified using a Bio-Rad Videodensitometer (Richmond, CA). Before rehybridization, membranes were washed at least 2 h at 65°C in 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05% sodium PPᵢ, and 0.1X Denhardt's solution.

Determination of ER Content. Cells were grown to subconfluence in 175-cm² culture flasks. Next, cells were washed 2 times in SFM during 24 h

to remove steroids. Cells were incubated with the vitamin D₃ compounds or vehicle (0.1% ethanol) in SFM for 24 or 48 h prior to harvesting with 3 mM EDTA in phosphate-buffered saline. Cell pellets were quickly frozen in liquid nitrogen and homogenized using a microdismembrator as described by Van Agthoven et al. (20). Cytosolic extracts were prepared by high-speed centrifugation at 100,000 × g for 20 min, and ER content was measured with an enzyme immunoassay (Abbott ER-EIA; Abbott Laboratories, Chicago, IL).

VDR Binding Assay. Cells were grown to subconfluence and washed for 24 h in SFM to remove steroids. A VDR binding assay was performed as described previously (26). Briefly, cells were harvested by trypsinization, and the cell pellets were extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 5 mM dithiotreitol, 10 mM sodium molybdate, and 0.1% Triton X-100 (pH 7.4). High-speed supernatants were obtained by centrifugation at 100,000 × g for 1 h at 4°C. Aliquots of cytosolic extracts (containing approximately 1 mg protein) were incubated for 3 h at 0°C with 0.25 nM [³H]1,25-(OH)₂D₃ and increasing concentrations (5 × 10⁻¹¹ M-5 × 10⁻⁷ M) of unlabeled 1,25-(OH)₂D₃ or vitamin D₃ analogue. Receptor-bound and free [³H]1,25-(OH)₂D₃ were separated by charcoal adsorption. The 1,25-(OH)₂D₃/analogue concentrations resulting in 50% displacement of bound [³H]1,25-(OH)₂D₃ were calculated.

RESULTS

Effect of Vitamin D₃ Compounds on the Growth of MCF-7 Cells. MCF-7 cells were able to grow exponentially in SFM without further additions (= autonomous growth). After an 8-day incubation period, DNA content of control cultures increased from 6 μg/well to about 50 μg/well. As shown in Fig. 2, 1,25-(OH)₂D₃ and the synthetic analogues OCT, CB966, EB1089, and KH1060 inhibited autonomous growth. The chemical structures of the analogues are depicted in Fig. 1. The maximum effect (25% inhibition) was similar for 1,25-(OH)₂D₃ and analogues and was reached at approximately 10⁻⁸ M EB1089 and KH1060, 10⁻⁷ M 1,25-(OH)₂D₃ and CB966, and 10⁻⁶ M OCT. The concentrations of the analogues needed to achieve the half-maximal effect of 1,25-(OH)₂D₃ (designated as EC₅₀) were assessed, and based on these concentrations, the relative potencies with respect to 1,25-(OH)₂D₃ were calculated. OCT displayed a somewhat decreased potency and CB966 a small increased potency, whereas EB1089 and KH1060 were clearly more potent than 1,25-(OH)₂D₃, 67 and 100 times, respectively (Table 1).

Since 17β-E₂ plays an important role in breast cancer development and growth, we have investigated the effect of the vitamin D₃ analogues on 17β-E₂-stimulated proliferation of MCF-7 cells. 17β-E₂ (10⁻¹⁰ M) resulted in a 2-fold stimulation of control cultures. Inhibition of 17β-E₂-stimulated growth by the vitamin D₃ compounds was relatively small (approximately 12%; data not shown). The absolute inhibition of 17β-E₂-stimulated growth expressed in μg DNA/well
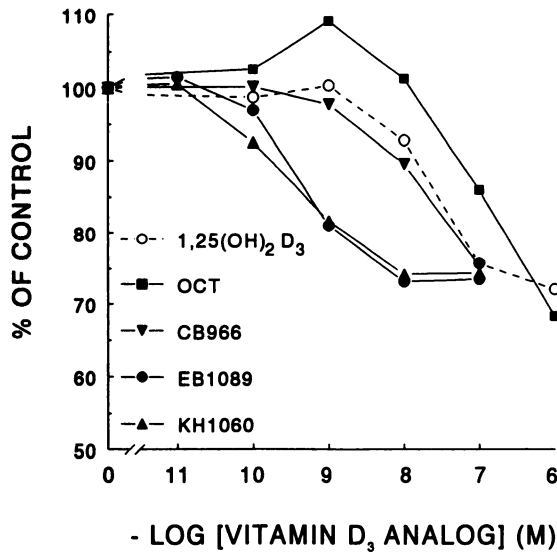
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Fig. 2. Inhibition of autonomous growth of MCF-7 cells by 1,25-(OH)$_2$D$_3$ and vitamin D$_3$ analogues. MCF-7 cells were cultured for 8 days in SFM as described in "Materials and Methods" in the absence or presence of various concentrations of the vitamin D$_3$ compounds. DNA content of the control cultures was set at 100% (49 μg DNA/well). Data represent the mean of three separate experiments, each consisting of duplicate wells.

sensitivity to 1,25-(OH)$_2$D$_3$/analogues was increased by combined treatment with tamoxifen, the inhibitory effect at the maximal effective concentrations of 1,25-(OH)$_2$D$_3$/analogues was additive.

Effect of Vitamin D$_3$ Compounds on the Growth of ZR-75-1 Cells. Next, we studied the effect of the vitamin D$_3$ compounds on another estrogen-responsive breast cancer cell line. As opposed to MCF-7 cells, ZR-75-1 cells are dependent on 17β-E$_2$ for growth. 17β-E$_2$ (10^{-10} M) induced an increase in DNA from 7 to 21 μg DNA/well in 8 days. Fig. 4 shows that, in contrast to 17β-E$_2$-stimulated growth of MCF-7 cells, 17β-E$_2$-stimulated growth of ZR-75-1 cells was potently inhibited by the vitamin D$_3$ compounds. The EC$_{50}$s for the growth inhibition of ZR-75-1 cells were similar to those of MCF-7 cells with the exception of EB1089, which had a somewhat reduced potency in ZR-75-1 cells (Table 2).

Analogous to MCF-7 cells, we have analyzed the combined effects of vitamin D$_3$ analogues and tamoxifen on the growth of ZR-75-1 cells. Tamoxifen (10^{-6} M) completely blocked 17β-E$_2$-stimulated growth of ZR-75-1 cells and, thereby, the entire proliferation. Therefore, we have used a suboptimal concentration of tamoxifen (10^{-7} M) to test whether there is an interaction between tamoxifen and vitamin D$_3$ compounds. Fig. 5 demonstrates that the inhibition by tamoxifen
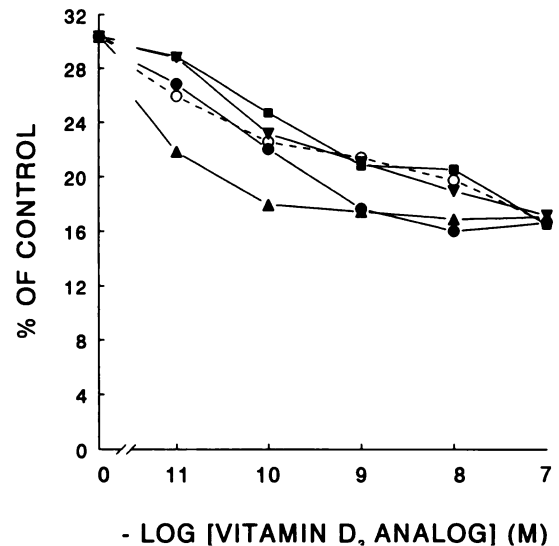
was similar to the absolute inhibition of the autonomous growth, indicating that there was no specific inhibition of the 17β-E$_2$ effect. Also, the EC$_{50}$s of 1,25-(OH)$_2$D$_3$ and analogues were in the same order of magnitude as for autonomous growth (Table 1).

Inhibition of 17β-E$_2$-stimulated growth of breast cancer cells by the antiestrogen tamoxifen is well documented. In MCF-7 cells, tamoxifen not only completely blocked 17β-E$_2$-stimulated growth but also inhibited part of the autonomous growth of MCF-7 cells, i.e., DNA values of cultures treated with 10^{-10} M 17β-E$_2$ and 10^{-6} M tamoxifen (30 μg/well) were lower than DNA values of control cultures (49 μg/well). Subsequently, we examined the effect of combined treatment with 1,25-(OH)$_2$D$_3$/analogues and tamoxifen on 17β-E$_2$-stimulated growth. Fig. 3 shows that, although tamoxifen alone causes a potent growth inhibition (reduction to 30% of 17β-E$_2$-stimulated growth), addition of 1,25-(OH)$_2$D$_3$ or analogues resulted in a still further inhibition (from 30% down to 17% of 17β-E$_2$-stimulated growth). In combination with tamoxifen, the EC$_{50}$s of 1,25-(OH)$_2$D$_3$ and analogues shifted to lower concentrations. The EC$_{50}$s of 1,25-(OH)$_2$D$_3$, CB966, and OCT were 200-, 125- and 100-fold lower, respectively, in the presence of tamoxifen, whereas the EC$_{50}$s of KH1060 and EB1089 were only 14- and 2-fold lower, respectively (Table 1). Consequently, the differences between 1,25-(OH)$_2$D$_3$ and the analogues are smaller in the presence of tamoxifen. Although the

Fig. 3. Inhibition of 17β-E$_2$-stimulated growth of MCF-7 cells by combined treatment with tamoxifen and vitamin D$_3$ compounds. Cells were cultured for 8 days in SFM supplemented with 10^{-10} M 17β-E$_2$ and 10^{-6} M tamoxifen, in the absence or presence of various concentrations of the vitamin D$_3$ compounds. Tamoxifen alone reduced 17β-E$_2$-stimulated proliferation to 30% (30 μg DNA/well), which is indicated as the starting value on the vertical axis. Data represent the mean of three separate experiments, each consisting of duplicate wells. Symbols, see Fig. 2.

Table 1 *Growth inhibition of MCF-7 cells by 1,25-(OH)$_2$D$_3$ and vitamin D$_3$ analogues and their affinity for the VDR*

	GROWTH INHIBITION							
	Autonomous growth		17β-E$_2$-stimulated growth				Ratio of EC$_{50}$ without and with tamoxifen[b]	VDR binding relative to 1,25-(OH)$_2$D$_3$
			Without tamoxifen		With tamoxifen			
Analogue	EC$_{50}$ (M)[a]	Relative to 1,25-(OH)$_2$D$_3$	EC$_{50}$ (M)	Relative to 1,25-(OH)$_2$D$_3$	EC$_{50}$ (M)	Relative to 1,25-(OH)$_2$D$_3$		
1,25-(OH)$_2$D$_3$	2×10^{-8}	1	1×10^{-8}	1	5×10^{-11}	1	200	1
OCT	8×10^{-8}	0.25	2×10^{-8}	0.50	2×10^{-10}	0.25	100	0.1
CB966	1×10^{-8}	2	1×10^{-8}	1	8×10^{-11}	0.63	125	0.7
EB1089	3×10^{-10}	67	1×10^{-10}	100	5×10^{-11}	1	2	0.7
KH1060	2×10^{-10}	100	1×10^{-10}	100	7×10^{-12}	7	14	0.7

[a] The concentrations of the analogues needed to achieve the half-maximal effect of 1,25-(OH)$_2$D$_3$ (designated as EC$_{50}$) were assessed on the basis of the data in Figs. 2 and 3. The maximum inhibition of autonomous growth was 25% and 17β-E$_2$-stimulated growth, 12%. Tamoxifen inhibited 17β-E$_2$-stimulated growth by 70%, and tamoxifen combined with the vitamin D$_3$ compounds resulted in a maximum inhibition of 83%.

[b] The ratio expresses the magnitude of the shift of the EC$_{50}$ by cotreatment with tamoxifen and was calculated by division of the EC$_{50}$ in the absence of tamoxifen by the EC$_{50}$ in the presence of tamoxifen. Binding of 1,25-(OH)$_2$D$_3$ and analogues to the VDR was measured in cytosolic extracts by displacement of [^{3}H]1,25-(OH)$_2$D$_3$.
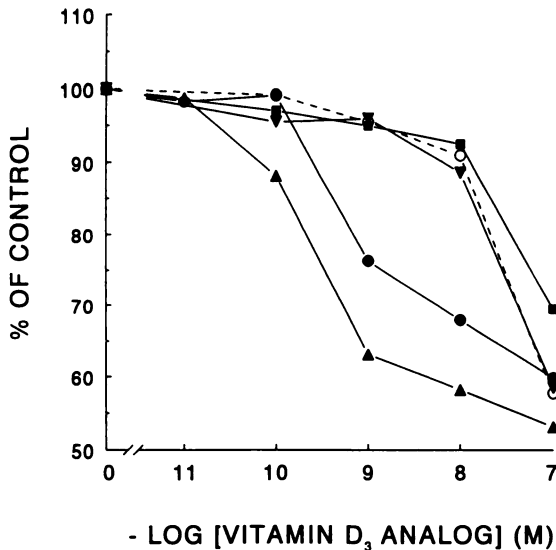
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Fig. 4. Growth inhibition of ZR-75-1 cells by 1,25-$(OH)_2D_3$ and vitamin D_3 analogues. ZR-75-1 cells were grown for 8 days in SFM supplemented with 10^{-10} M 17β-E_2 in the presence or absence of various concentrations of the vitamin D_3 compounds. DNA values of control cultures (no vitamin D added) increased from 7 to 21 μg DNA/well in 8 days. The absolute increase in DNA content (14 μg DNA/well) was set at 100%. Data represent the mean of three separate experiments, each consisting of duplicate wells. Symbols, see Fig. 2.

Table 2 *Growth inhibition of ZR-75-1 cells by 1,25-$(OH)_2D_3$ and vitamin D_3 analogues*

| Analogue | 17β-E_2-stimulated growth | | | | Ratio of EC_{50} without and with tamoxifen[b] |
| | Without tamoxifen | | With tamoxifen | | |
	EC_{50} (M)[a]	Relative to 1,25-$(OH)_2D_3$	EC_{50} (M)	Relative to 1,25-$(OH)_2D_3$	
1,25-$(OH)_2D_3$	2×10^{-8}	1	1×10^{-9}	1	20
OCT	4×10^{-8}	0.5	1×10^{-11}	100	4000
CB966	2×10^{-8}	1	1×10^{-9}	1	20
EB1089	7×10^{-10}	29	2×10^{-10}	5	4
KH1060	2×10^{-10}	100	2×10^{-11}	50	10

[a] The concentrations of the analogue needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ (designated as EC_{50}) were assessed on the basis of the data in Figs. 4 and 5. The maximal inhibition of the 17β-E_2-stimulated growth was 40%. Tamoxifen inhibited the 17β-E_2-stimulated growth 51%, and tamoxifen combined with the vitamin D_3 compounds resulted in a maximum inhibition of 79%.

[b] The ratio expresses the magnitude of the shift of the EC_{50} by cotreatment with tamoxifen and was calculated by division of the EC_{50} in the absence of tamoxifen by the EC_{50} in the presence of tamoxifen.

(reduction to 49% of 17β-E_2-stimulated growth) can be augmented by addition of 1,25-$(OH)_2D_3$ or analogues (further reduction from 49% down to an average of 21% of 17β-E_2-stimulated growth). Similar to MCF-7 cells, the EC_{50}s for the inhibition by the vitamin D_3 compounds were shifted to the left in the presence of tamoxifen. The EC_{50} of OCT was even 4000 times lower; the EC_{50}s of 1,25-$(OH)_2D_3$ and CB966, 20 times; and the EC_{50}s of EB1089 and KH1060, 4 and 10 times; respectively (Table 2). Despite the increased sensitivity of 1,25-$(OH)_2D_3$ and analogues, the effect of combined treatment with tamoxifen was less than additive at the maximal effective concentrations of the vitamin D_3 compounds.

To assess whether treatment with 1,25-$(OH)_2D_3$/analogues could be useful in an antiestrogen-resistant situation, we have tested the effect of 1,25-$(OH)_2D_3$ and KH1060 on tamoxifen-resistant derivatives of ZR-75-1 cells. These resistant cells were cloned from estrogen-dependent ZR-75-1 cells subjected to tamoxifen selection after retrovirus infection (19). Table 3 demonstrates that the growth of the resistant XI 13 and VIII 24 cells was indeed inhibited by 1,25-$(OH)_2D_3$ and KH1060, with KH1060 being more potent. Furthermore,

EGF-dependent proliferation of EGF receptor-positive derivatives of ZR-75-1 cells (20) was strongly inhibited by 1,25-$(OH)_2D_3$ and KH1060 (Table 3).

Regulation of ER Content. To study whether vitamin D_3 compounds exert their effects on proliferation via regulation of ER level, we have measured ER content of both cell lines using an enzyme immunoassay. The vitamin D_3 compounds 1,25-$(OH)_2D_3$, CB966, EB1089, KH1060, and OCT (10^{-8} M) had no effect on the ER level of MCF-7 (750 fmol/mg protein) and ZR-75-1 cells (180 fmol/mg protein) after 24 and 48 h of incubation (data not shown).

Regulation of c-*myc* mRNA Expression. To study a possible involvement of c-*myc* in the growth inhibition by vitamin D_3 compounds, we have studied c-*myc* mRNA expression in MCF-7 and ZR-75-1 cells. In an attempt to relate growth inhibition to effects on c-*myc* mRNA expression, we have measured c-*myc* under the same conditions as the proliferation experiments. c-*myc* mRNA levels of autonomously growing MCF-7 cells incubated with 10^{-7} M 1,25-$(OH)_2D_3$ for 0.5 up to 48 h did not change significantly with respect to the control incubation (vehicle added; data not shown). 17β-E_2-stimulated growth of MCF-7 and ZR-75-1 cells was studied using 10^{-10} M 17β-E_2. At this concentration, c-*myc* was rapidly induced in
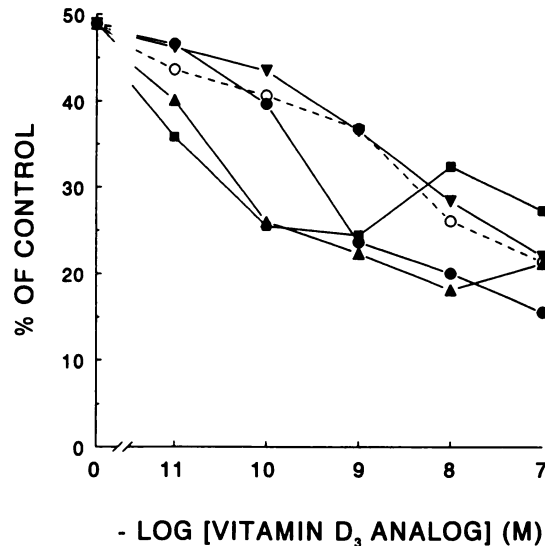

Fig. 5. Growth inhibition of ZR-75-1 cells by combined treatment with tamoxifen and vitamin D_3 compounds. Cells were cultured for 8 days in SFM supplemented with 10^{-10} M 17β-E_2 and 10^{-7} M tamoxifen, in the absence or presence of various concentrations of the vitamin D_3 compounds. DNA content was measured and corrected for DNA content at the start of the experiment. Tamoxifen alone reduced 17β-E_2-induced proliferation to 49%, which is indicated as the starting value on the *vertical axis*. Data represent the mean of three separate experiments, each consisting of duplicate wells. Symbols, see Fig. 2.

Table 3 *Growth inhibition of tamoxifen-resistant and EGF receptor-positive derivatives of ZR-75-1 cells by 1,25-$(OH)_2D_3$ and KH1060*

Cells were cultured in basal RPMI medium supplemented with 10% FCS in the absence or presence of 1,25-$(OH)_2D_3$ or KH1060. 17β-E_2 (10^{-10} M) was added in cultures of parental ZR-75-1 cells and EGF (10 ng/ml) in cultures of EGF receptor-positive ZR/HERc cells to induce the growth of these cells. The tamoxifen-resistant XI 13 and VIII 24 cells are able to grow autonomously. Proliferation of XI 13 cells was measured after 5 days, and proliferation of ZR-75-1, VIII 24, and ZR/HERc cells was measured after 7 days by crystal violet absorbance. Data are expressed as the percentage of control and represent mean ± SD of nine wells.

| Condition | % of control | | | |
	ZR-75-1	XI 13	VIII 24	ZR/HERc
Control	100 ± 6	100 ± 3	100 ± 3	100 ± 9
10^{-9} M 1,25-$(OH)_2D_3$	107 ± 9	113 ± 3	88 ± 4	98 ± 6
10^{-7} M 1,25-$(OH)_2D_3$	55 ± 8	78 ± 3	35 ± 1	30 ± 7
10^{-9} M KH1060	59 ± 6	80 ± 4	37 ± 3	29 ± 7
10^{-7} M KH1060	49 ± 4	79 ± 3	35 ± 3	28 ± 7

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Fig. 6. Northern analysis of c-myc in MCF-7 cells. Autoradiograph of RNA hybridized with ^{32}P-labeled c-myc and GAPDH probes. GAPDH was used to check equal loading of the lanes and rRNA bands (28S and 18S) served as molecular weight markers. Cells were cultured as described in "Materials and Methods" and incubated for 1 h with vehicle (Lanes 1 and 18), 10^{-10} M 17β-E₂ (lanes 2–21), or 10^{-6} M tamoxifen (Lanes 18–21). 1,25-(OH)₂D₃ (10^{-9}, 10^{-8}, and 10^{-7} M) was added in Lanes 3–5 and Lanes 19–21, CB966 in Lanes 6–8, EB1089 in Lanes 9–11, KH1060 in Lanes 12–14, and OCT in Lanes 15–17.
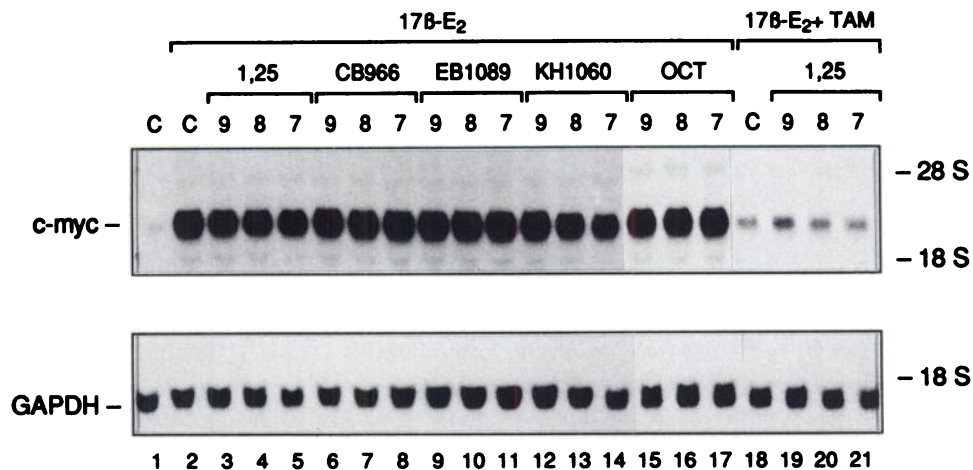
MCF-7 cells (25-fold) and ZR-75-1 cells (2-fold), with a peak at 1 h and a gradual return towards prestimulation level in 4 h. In MCF-7 cells, 10^{-10} M 17β-E₂ was the maximal effective concentration, whereas in ZR-75-1 cells, a maximal 6-fold stimulation was achieved at 10^{-9} M 17β-E₂. Fig. 6 demonstrates that neither 1,25-(OH)₂D₃ nor vitamin D₃ analogues modulated the 17β-E₂-induced c-myc mRNA expression in MCF-7 cells (Fig. 6, Lanes 2–17). Tamoxifen (10^{-6} M) almost completely inhibited c-myc induction by 17β-E₂ (Fig. 6, Lane 18). With ZR-75-1 cells, similar results were obtained (data not shown). Subsequently, we have tested in MCF-7 cells the combined effects of 1,25-(OH)₂D₃/analogues and tamoxifen. The inhibition of 17β-E₂-induced c-myc expression by tamoxifen was neither augmented by combined treatment with 1,25-(OH)₂D₃ (Fig. 6, Lanes 19–21) nor by the vitamin D₃ analogues (data not shown).

VDR Binding. VDR binding of the analogues was measured to study whether the differences between the vitamin D₃ analogues in their ability to inhibit breast cancer cell growth were related to their affinity for the VDR. It is demonstrated in Table 1 that the analogues had a lower affinity for the VDR compared to 1,25-(OH)₂D₃. The reduced growth inhibitory potential of OCT corresponded with a lower VDR affinity compared to 1,25-(OH)₂D₃, but the increased growth inhibitory potential of EB1089 and KH1060 did not correspond with their lower VDR affinity.

To study whether the increased sensitivity to the vitamin D₃ compounds by cotreatment with tamoxifen was caused by increased VDR binding, we have measured VDR concentration in MCF-7 cells after incubation for 24 h with 10^{-6} M tamoxifen. The VDR concentration of 15 fmol/mg protein was not changed by treatment with tamoxifen. Also, 17β-E₂ had no effect on VDR levels.

DISCUSSION

The present data show that the growth inhibitory action of vitamin D₃ compounds and tamoxifen are complementary. In MCF-7 cells, which have partially escaped from estrogenic control, combined treatment resulted in a stronger inhibition than treatment with either compound alone. In ZR-75-1 cells, which are fully estrogen dependent, the entire proliferation could be blocked by a high concentration of tamoxifen, whereas at lower tamoxifen concentration, vitamin D₃ compounds were able to augment the inhibitory effect of tamoxifen, similar as in MCF-7 cells. The clinical use of lower dosages of tamoxifen may be beneficial, considering the relationship between tamoxifen and an increased risk on endometrial cancer (16). Resistance to tamoxifen therapy frequently occurs (17), and in this situation, treatment with vitamin D₃ analogues could also be useful. This is

supported by our data that derivatives of ZR-75-1 cells, which had acquired resistance to tamoxifen as a result of retroviral insertional mutagenesis (19), had not lost their response to the growth-inhibitory action of the vitamin D₃ compounds.

Despite the promising antiproliferative effects in vitro, the calcemic effects of vitamin D₃ compounds may result in the development of hypercalcemia when applied in vivo. The present data show that the analogues EB1089 and KH1060 have the same growth-inhibitory action as 1,25-(OH)₂D₃ at up to 100-fold lower concentrations, whereas in vivo studies have shown that the calcemic activity of these compounds was equal or even lower (8, 9). These results support the idea that the antiproliferative effects can be (partly) dissociated from the calcemic effects. Our data on the potent growth inhibitory effects of EB1089 and KH1060 are in line with other reports (8, 12, 13). Additionally, we show for the first time that EB1089 and KH1060 can also inhibit the growth of a fully 17β-E₂-dependent cell line (ZR-75–1) with increased potency compared to 1,25-(OH)₂D₃. In various ER-positive and ER-negative human breast cancer cell lines, OCT was 10 times more potent than 1,25-(OH)₂D₃ (11). In our hands, the potency of OCT was somewhat diminished compared to 1,25-(OH)₂D₃ in both cell lines studied. The reason for this discrepancy is not yet known. CB966 and 1,25-(OH)₂D₃ were equipotent, and to our knowledge there are no other reports on the effect of CB966 on breast cancer cells.

For translation of the antiproliferative action of vitamin D₃ analogues on breast cancer cells in culture to the in vivo situation, the pharmacokinetic properties of the analogues play an important role (27). Furthermore, negative side-effects have to be monitored. Apart from the development of hypercalcemia, other effects may arise. For instance, the oxa-compounds OCT and KH1060 have been shown to exert strong immunosuppressive activity (8, 28), and stimulation of bone resorption might increase the incidence of skeletal metastases (29).

In view of the clinical importance of using low doses, the observation that, in combination with tamoxifen, the EC₅₀s of 1,25-(OH)₂D₃ and analogues shifted to lower concentrations might be of interest. This shift was more pronounced for 1,25-(OH)₂D₃ and the compounds with an EC₅₀ close to 1,25-(OH)₂D₃ (OCT and CB966) than for the compounds with a more favorable EC₅₀ (EB1089 and KH1060) in the absence of tamoxifen. Thereby, the differences in potencies between the analogues were smaller in the presence of tamoxifen. The shift of the EC₅₀s of the vitamin D₃ compounds in the presence of tamoxifen points to an interaction between both types of growth inhibitors. This is in agreement with a report from Abe-Hashimoto et al. (30), who have observed synergism between OCT

and tamoxifen in MCF-7 and ZR-75-1 cells *in vitro* as well as in MCF-7 tumor *in vivo*. The mechanism of the interaction between tamoxifen and vitamin D_3 compounds is yet unclear, but the present data show that it was not achieved via a change in VDR level. Notwithstanding the interaction between both growth inhibitors, at the maximal effective concentrations of the vitamin D_3 compounds the effect of combined treatment with tamoxifen was additive (MCF-7 cells) or less than additive (ZR-75-1 cells).

Our data on the effect of combined treatment with tamoxifen and vitamin D_3 compounds point to several potential advantages when applied *in vivo*: (*a*) a more beneficial response can be achieved than by either agent alone; (*b*) lower concentrations of 1,25-$(OH)_2D_3$/ analogues and tamoxifen can be used with reduced risk of negative side-effects (hypercalcemia, increased bone turnover, and endometrial cancer); (*c*) since tumors are believed to be heterogeneous with respect to ER status (31), combination therapy may have the advantage that both ER-positive and ER-negative cells are inhibited; (*d*) tumor flare in response to tamoxifen may be prevented since 1,25-$(OH)_2D_3$ has been shown to inhibit the estrogenic effect of tamoxifen (18); and (*e*) tamoxifen may attenuate the negative effects of vitamin D_3 compounds on bone metabolism by its positive estrogenic effect on bone (32).

Since 17β-E_2 is an important regulator of breast cancer growth, we have addressed the question whether vitamin D_3 compounds can interfere directly with the growth stimulation by 17β-E_2. The data obtained with MCF-7 cells clearly indicate an estrogen-independent mechanism of action: (*a*) the inhibition of 17β-E_2-stimulated growth, expressed in μg DNA/well, by the vitamin D_3 compounds was similar to the inhibition of autonomous growth (maximum and EC_{50}s); (*b*) the ER level was not down-regulated; (*c*) the rapid induction of c-*myc* mRNA, which is thought to mediate the growth effect of 17β-E_2 (33) was not affected; and (*d*) the expression of the 17β-E_2-regulated pS2 gene was not changed.[3] Moreover, an estrogen-independent mechanism of action is consistent with the fact that 1,25-$(OH)_2D_3$ or analogues inhibit breast cancer cell growth, irrespective of the presence of the ER (4, 11). The inhibition of 17β-E_2-induced growth of ZR-75-1 cells seems to contradict an entirely 17β-E_2-independent mechanism of action of the vitamin D_3 compounds. However, as in MCF-7 cells, the ER and c-*myc* levels were not affected. Furthermore, EGF-dependent proliferation of ZR/HERc cells (derived from ZR-75-1) was also inhibited, indicating that vitamin D_3 compounds affect ZR-75-1 cell growth at a late stage of the signal cascades used by both the ER and EGF receptor. This may be achieved by induction of a negative growth factor or the inhibition of an autocrine loop. Also, the observed interaction between vitamin D_3 compounds and tamoxifen may occur at this level, since tamoxifen has been shown to exert antiestrogenic as well as antigrowth factor activity mediated via the ER (34).

In MCF-7 cells and several other cell types, it has been demonstrated that 1,25-$(OH)_2D_3$-induced differentiation and growth inhibition was preceded by a decrease in mRNA of the proto-oncogene c-*myc* (13, 35–37), indicating that c-*myc* gene regulation could be involved in the pathway for 1,25-$(OH)_2D_3$-mediated control of cell proliferation. However, we did not find changes in c-*myc* mRNA by 1,25-$(OH)_2D_3$, neither in autonomously growing MCF-7 cells nor in 17β-E_2-stimulated MCF-7 and ZR-75-1 cells, suggesting that down-regulation of c-*myc* mRNA is not a prerequisite for the growth inhibition by vitamin D_3 compounds in breast cancer cells.

The differences between the growth inhibitory potential of the analogues are difficult to explain. The present data show that the

maximal inhibition by 1,25-$(OH)_2D_3$ and analogues was the same, suggesting that they all act via the same pathway. Measurements of VDR affinity showed that the increased growth inhibitory potential of EB1089 and KH1060 was not related to a higher VDR affinity. Also, VDR-independent or nongenomic mechanisms of action have been attributed to 1,25-$(OH)_2D_3$ and analogues (2). However, it has been reported that the antiproliferative action of 1,25-$(OH)_2D_3$/analogues is restricted to VDR-positive cells (38). Other explanations could be differences in cellular metabolism of the analogues, receptor phosphorylation, or conformational changes of the receptor-ligand complex.

In conclusion, a more beneficial growth response of ER-positive breast cancer cells was observed by combined treatment with vitamin D_3 analogues and tamoxifen than by treatment with these compounds alone. The vitamin D_3 analogues were active at up to 100-fold lower concentrations than the native compound 1,25-$(OH)_2D_3$, which is important to reduce the risk on negative side-effects. Tamoxifen increased the sensitivity to 1,25-$(OH)_2D_3$ and analogues, which could implicate that in combination therapy even lower concentrations of the vitamin D_3 compounds can be used. Furthermore, it was shown that also the growth of antiestrogen-resistant cells could be inhibited by 1,25-$(OH)_2D_3$ and analogues. Thereby, vitamin D_3 analogues are interesting candidates for breast cancer therapy.

ACKNOWLEDGMENTS

REFERENCES

1. Reichel, H., Koeffler, H. P., and Norman, A. W. The role of the vitamin D endocrine system in health and disease. N. Engl. J. Med., *320:* 980–991, 1989.
2. Walters, M. R. Newly identified actions of the vitamin D endocrine system. Endocr. Rev., *13:* 719–764, 1992.
3. Frampton, R. J., Omond, S. A., and Eisman, J. A. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D₃ metabolites. Cancer Res., *43:* 4443–4447, 1983.
4. Chouvet, C., Vicard, E., Devonec, M., and Saez, S. 1,25-Dihydroxyvitamin D₃ inhibitory effect on the growth of two human breast cancer cell lines (MCF-7, BT-20). J. Steroid Biochem., *24:* 373–376, 1986.
5. Iino, Y., Yoshida, M., Sugamata, N., Maemura, M., Ohwada, S., Yokoe, T., Ishikita, T., Horiuchi, R., and Morishita, Y. 1α-Hydroxyvitamin D₃, hypercalcemia, and growth suppression of 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumors. Breast Cancer Res. Treat., *22:* 133–140, 1992.
6. Pols, H. A. P., Birkenhäger, J. C., Foekens, J. A., and Van Leeuwen, J. P. T. M. Vitamin D: a modulator of cell proliferation and differentiation. J. Steroid Biochem. Mol. Biol., *6:* 873–876, 1990.
7. Abe, J., Morikawa, M., Miyamoto, K., Kaiho, S., Fukushima, M., Miyaura, C., Abe, E., Suda, T., and Nishii, Y. Synthetic analogues of vitamin D₃ with an oxygen atom in the side chain skeleton. FEBS Lett., *226:* 58–62, 1987.
8. Binderup, L., Latini, S., Binderup, E., Bretting, C., Calverley, M., and Hansen, K. 20-Epi-vitamin D₃ analogues: a novel class of potent regulators of cell growth and immune responses. Biochem. Pharmacol., *42:* 1569–1575, 1991.
9. Binderup, E., Calverley, M. J., and Binderup, L. Synthesis and biological activity of 1α-hydroxylated vitamin D analogues with polyunsaturated side chains. *In:* A. W. Norman, R. Bouillon, and M. Thomasset (eds.), Vitamin D. Gene regulation, Structure-Function Analysis and Clinical Application, pp. 192–193. Berlin, Germany: Walter de Gruyter, 1991.
10. Sato, K., Nishii, Y., Woodiel, F. N., and Raisz, L. G. Effects of two new vitamin D₃ derivatives, 22-oxa-1α-25-dihydroxyvitamin D₃ (OCT) and 2β-(3-hydroxypropoxy)-1α,25-dihydroxyvitamin D₃ (ED-71), on bone metabolism in organ culture. Bone, *14:* 47–51, 1993.
11. Abe, J., Nakano, T., Nishii, Y., Matsumoto, T., Ogata, E., and Ikeda, K. A novel vitamin D₃ analog, 22-oxa-1,25-dihydroxyvitamin D₃, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. Endocrinology, *129:* 832–837, 1991.
12. Colston, K. W., Mackay, A. G., James, S. Y., and Binderup, L. EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. Biochem. Pharmacol., *44:* 2273–2280, 1992.
13. Mathiasen, I. S., Colston, K. W., Binderup, L. EB1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. J. Steroid Biochem. Mol. Biol., *46:* 365–371, 1993.
14. Eisman, J. A., Suva, L. J., Sher, E., Pearce, P. J., Funder, J. W., and Martin, T. J. Frequency of 1,25-dihydroxyvitamin D₃ receptor in human breast cancer. Cancer Res., *41:* 5121–5124, 1981.

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[3] T. Vink-van Wijngaarden, H. A. P. Pols, J. C. Birkenhäger, and J. P. T. M. van Leeuwen, unpublished data.

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15. Berger, U., Wilson, P., McClelland, R. A., Colston, K., Haussler, M. R., Pike, J. W., and Coombes, R. C. Immunocytochemical detection of 1,25-dihydroxyvitamin D₃ receptor in breast cancer. Cancer Res., 47: 6793–6799, 1987.

16. Jordan, V. C. A current view of tamoxifen for the treatment and prevention of breast cancer. Br. J. Pharmacol., 110: 507–517, 1993.

17. Jordan, V. C., Robinson, S. P., and Welshons, W. V. Resistance to antiestrogen therapy. In: D. Kessel (ed.), Resistance to Antineoplastic Drugs, pp. 403–427. Boca Raton: CRC Press, 1989.

18. Vink-van Wijngaarden, T., Pols, H. A. P., Buurman, C. J., Birkenhäger, J. C., and Van Leeuwen, J. P. T. M. Combined effects of 1,25-dihydroxyvitamin D₃ and tamoxifen on the growth of MCF-7 and ZR-75-1 human breast cancer cells. Breast Cancer Res. Treat., 29: 161–168, 1993.

19. Dorssers, L. C. J., Van Agthoven, T., Dekker, A., Van Agthoven, T. L. A., and Kok, E. M. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. Mol. Endocrinol., 7: 870–878, 1993.

20. Van Agthoven, T., Van Agthoven, T. L. A., Portengen, H., Foekens, J. A., and Dorssers, L. C. J. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. Cancer Res., 52: 5082–5088, 1992.

21. Westergren-Thorsson, G., Önnervik, P. O., Fransson, L. å., and Malmström, A. Proliferation of cultured fibroblasts is inhibited by L-induronate-containing glycosaminoglycans. J. Cell. Physiol., 147: 523–530, 1991.

22. Chomczinski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156–159, 1987.

23. Davis, L. G., Dibner, M. D., and Battey, J. F. Formaldehyde gel for electrophoretic separation of RNA and Northern blot. In: Basic Methods in Molecular Biology, pp. 143–149. Amsterdam, the Netherlands: Elsevier, 1986.

24. Dalla-Favera, R., Gelmann, E. P., Martinotti, S., Franchini, G., Papas, T. S., Gallo, R. C., and Wong-Staal, F. Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA, 79: 6497–6501, 1982.

25. Benham, F. J., Hodgkinson, S., and Davies, K. E. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosome defines a multigene family. EMBO J., 3: 2635–2640, 1984.

26. Pols, H. A. P., Birkenhäger, J. C., Schilte, J. P., Bos, M. P., and Van Leeuwen, J. P. T. M. The effects of MC903 on 1,25-(OH)₂D₃ receptor binding, 24-hydroxylase activity and in vitro bone resorption. Bone Miner., 14: 103–111, 1991.

27. Pols, H. A. P., Birkenhäger, J. C., and Van Leeuwen, J. P. T. M. Vitamin D analogues: from molecule to clinical application. Clin. Endocrinol., 40: 285–291, 1994.

28. Abe, J., Nakamura, K., Takita, Y., Nakano, T., Irie, H., and Nishii, Y. Prevention of immunological disorders in MRL/I mice by a new synthetic analogue of vitamin D₃: 22-oxa-1α,25-dihydroxyvitamin D₃. J. Nutr. Sci. Vitaminol., 36: 21–31, 1990.

29. Orr, F. W., Kostenuik, P., Sanchez-Sweatman, O. H., and Singh, G. Mechanisms involved in the metastasis of cancer to bone. Breast Cancer Res. Treat., 25: 151–163, 1993.

30. Abe-Hashimoto, J., Kikuchi, T., Matsumoto, T., Nishii, Y., Ogata, E., and Ikeda, K. Antitumor effect of 22-oxa-calcitriol, a noncalcemic analogue of calcitriol, in athymic mice implanted with human breast carcinoma and its synergism with tamoxifen. Cancer Res., 53: 2534–2537, 1993.

31. Osborne, C. K. Heterogeneity in hormone receptor status in primary and metastatic breast cancer. Semin. Oncol., 12: 317–326, 1985.

32. Wright, C. D. P., Garrahan, N. J., Stanton, M., Gazet, J. C., Mansell, R. E., Compston, J. E. Effect of long-term tamoxifen therapy on cancellous bone remodeling and structure in women with breast cancer. J. Bone Miner. Res., 9: 153–159, 1994.

33. Shiu, R. P. C., Watson, P. H., and Dubik, D. c-Myc oncogene expression in estrogen-dependent and -independent breast cancer. Clin. Chem., 39: 353–355, 1993.

34. Freiss, G., Prebois, C., Rochefort, H., and Vignon, F. Anti-steroidal and anti-growth factor activities of anti-estrogens. J. Steroid Biochem. Mol. Biol., 37: 777–781, 1990.

35. Reitsma, P. H., Rothberg, P. G., Astrin, S. M., Trial, J., Bar-Shavit, Z., Hall, A., Teitelbaum, S. L., and Kahn, A. J. Regulation of myc gene expression in HL-60 leukaemia cells by a vitamin D metabolite. Nature (Lond.), 306: 492–494, 1983.

36. Matsumoto, K., Hashimoto, K., Nishida, Y., Hashiro, M., and Yoshikawa, K. Growth-inhibitory effects of 1,25-dihydroxyvitamin D₃ on normal human keratinocytes cultured in serum-free medium. Biochem. Biophys. Res. Commun., 166: 916–923, 1990.

37. Kremer, R., Bolivar, I., Goltzman, D., and Hendy, G. N. Influence of calcium and 1,25-dihydroxycholecalciferol on proliferation and proto-oncogene expression in primary cultures of bovine parathyroid cells. Endocrinology, 125: 935–941, 1989.

38. Eisman, J. A., Barkla, D. H., and Tutton, P. J. M. Suppression of in vivo growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D₃. Cancer Res., 47: 21–25, 1987.