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 antimutagen, 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, 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. Bindener 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...
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. Cells were allowed to attach for 24 h, 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, 0.2 mM EDTA (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05% sodium PPO, and 0.1X Denhardt’s solution].

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 PPO, 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 microdismembranator 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 dithioreitol, 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 to 5 × 10⁻⁹ 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...
was similar to the absolute inhibition of the autonomous growth, indicating that there was no specific inhibition of the 17β-E₂ effect.

Also, the EC₅₀ values of cultures treated with 10⁻¹⁰ M 17β-E₂ and 10⁻⁶ M tamoxifen were only 14- and 2-fold lower, respectively, in the presence of tamoxifen, whereas the EC₅₀ values of KH1060 and EB1089 were only 14- and 2-fold lower, respectively (Table 1). Consequently, the differences between 1,25-(OH)₂D₃ and the analogues are smaller in the presence of tamoxifen. Although the sensitivity to 1,25-(OH)₂D₃/analogues was increased by combined treatment with tamoxifen, the inhibitory effect at the maximal effective concentrations of 1,25-(OH)₂D₃/analogues was additive.

Table 1 Growth inhibition of MCF-7 cells by 1,25-(OH)₂D₃ and vitamin D₃ analogues and their affinity for the VDR

<table>
<thead>
<tr>
<th>Analogue</th>
<th>17β-E₂-stimulated growth Without tamoxifen</th>
<th>17β-E₂-stimulated growth With tamoxifen</th>
<th>Ratio of EC₅₀ without and with tamoxifen</th>
<th>VDR binding relative to 1,25-(OH)₂D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-(OH)₂D₃</td>
<td>2 x 10⁻⁸</td>
<td>2 x 10⁻¹⁰</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>OCT</td>
<td>8 x 10⁻⁸</td>
<td>2 x 10⁻¹⁰</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CB966</td>
<td>1 x 10⁻⁸</td>
<td>1 x 10⁻¹⁰</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EB1089</td>
<td>1 x 10⁻¹⁰</td>
<td>1 x 10⁻¹⁰</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KH1060</td>
<td>2 x 10⁻¹⁰</td>
<td>1 x 10⁻¹⁰</td>
<td>100</td>
<td>100</td>
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</tbody>
</table>
with tamoxifen was less than additive at the maximal effective
levels. These resistant cells were cloned from estrogen-dc
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-
stimulated growth of MCF-7 and ZR-75-1 cells was studied using
EB1089, KH1O6O, and OCT (10^{-8} M) had no effect on the ER level
of parental ZR-75-1 cells and EGF (10 ng/ml) in cultures of EGF receptor-positive
derivatives of ZR-75-1 cells (20) was strongly inhibited by 1,25-(OH)_{2}D_{3}
and KH1O6O (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)_{2}D_{3}, CB966,
EB1089, KH1O6O, 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-
stimulated growth of MCF-7 and ZR-75-1 cells was studied using
10^{-10} M 17B-E_{2}. At this concentration, c-myc was rapidly induced in

(reduction to 49% of 17B-E_{2}-stimulated growth) can be augmented by
addition of 1,25-(OH)_{2}D_{3} or analogues (further reduction from 49% down
to an average of 21% of 17B-E_{2}-stimulated growth). Similar to
MCF-7 cells, the EC_{50} 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} of 1,25-(OH)_{2}D_{3}
and CB966, 20 times; and the EC_{50} of EB1089 and KH1O6O, 4 and 10
times, respectively (Table 2). Despite the increased sensitivity of
1,25-(OH)_{2}D_{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)_{2}D_{3} or analogues
could be useful in an antiestrogen-resistant situation, we have tested the effect of
1,25-(OH)_{2}D_{3} and KH1O6O on tamoxifen-resistant derivatives of
ZR-75-1 cells. These resistant cells were cloned from estrogen-dep
endent 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)_{2}D_{3}
and KH1O6O, with KH1O6O 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)_{2}D_{3} and
KH1O6O (Table 3).

Table 2 Growth inhibition of ZR-75-1 cells by 1,25-(OH)_{2}D_{3} and vitamin D_{3}
analogues

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Without tamoxifen</th>
<th>With tamoxifen</th>
<th>Ratio of EC_{50} with and without tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-(OH)<em>{2}D</em>{3}</td>
<td>2 \times 10^{-8}</td>
<td>1</td>
<td>1 \times 10^{-9}</td>
</tr>
<tr>
<td>OCT</td>
<td>4 \times 10^{-8}</td>
<td>0.5</td>
<td>1 \times 10^{-11}</td>
</tr>
<tr>
<td>CB966</td>
<td>2 \times 10^{-8}</td>
<td>1</td>
<td>1 \times 10^{-9}</td>
</tr>
<tr>
<td>EB1089</td>
<td>7 \times 10^{-10}</td>
<td>29</td>
<td>2 \times 10^{-10}</td>
</tr>
<tr>
<td>KH1O6O</td>
<td>2 \times 10^{-10}</td>
<td>100</td>
<td>2 \times 10^{-11}</td>
</tr>
</tbody>
</table>

The concentrations of the analogue 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. 4 and
5. The maximal inhibition of the 17B-E_{2}-stimulated growth was 40%. Tamoxifen inhibi
ted the 17B-E_{2}-induced proliferation 51%, and tamoxifen combined with the vitamin D_{3}
compounds resulted in a maximum inhibition of 79%.

The ratio expresses the magnitude of the shift of the EC_{50} by cotreatment with
tamoxifen. Without tamoxifen, with tamoxifen.

Fig. 4. Growth inhibition of ZR-75-1 cells by 1,25-(OH)_{2}D_{3} and vitamin D_{3}
analogues.

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 17B-E_{2} and 10^{-7} M tamoxifen, in the absence or presence of various concen
trations of the vitamin D_{3} compounds. DNA content was measured and corrected for DNA
content at the start of the experiment. Tamoxifen alone reduced 17B-E_{2}-induced prolif
eration 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>
<thead>
<tr>
<th>Condition</th>
<th>ZR-75-1</th>
<th>XI 13</th>
<th>VIII 24</th>
<th>ZR/HERc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 6</td>
<td>100 ± 3</td>
<td>100 ± 3</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>10^{-7} M 1,25-(OH)<em>{2}D</em>{3}</td>
<td>107 ± 9</td>
<td>113 ± 3</td>
<td>88 ± 4</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>10^{-7} M 1,25-(OH)<em>{2}D</em>{3}</td>
<td>55 ± 8</td>
<td>78 ± 3</td>
<td>35 ± 1</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>10^{-7} M KH1O6O</td>
<td>59 ± 6</td>
<td>80 ± 4</td>
<td>37 ± 3</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>10^{-7} M KH1O6O</td>
<td>49 ± 4</td>
<td>79 ± 3</td>
<td>35 ± 3</td>
<td>28 ± 7</td>
</tr>
</tbody>
</table>

Table 3 Growth inhibition of tamoxifen-resistant and EGF receptor-positive derivatives
of ZR-75-1 cells by 1,25-(OH)_{2}D_{3} and KH1O6O

Cells were cultured in basal RPMI medium supplemented with 10% FCS in the
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 17B-E_{2}-induced prolif
eration 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.
MC7-7 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 MC7-7 cells, 10^{-10} M 17B-E_2 was the maximal effective concentration, whereas in ZR-75-1 cells, a maximal 6-fold stimulation was achieved at 10^{-9} M 17B-E_2. Fig. 6 demonstrates that neither 1,25-(OH)_2D_3 nor vitamin D analogues modulated the 17B-E_2-induced c-myc mRNA expression in MC7-7 cells (Fig. 6, Lanes 2-17). Tamoxifen (10^{-6} M) almost completely inhibited c-myc induction by 17B-E_2 (Fig. 6, Lane 18). With ZR-75-1 cells, similar results were obtained (data not shown). Subsequently, we have tested in MC7-7 cells the combined effects of 1,25-(OH)_2D_3 analogues and tamoxifen. The inhibition of 17B-E_2-induced c-myc expression by tamoxifen was neither augmented by combined treatment with 1,25-(OH)_2D_3 (Fig. 6, Lanes 19-21) nor by the vitamin D analogues (data not shown).

**VDR Binding.** VDR binding of the analogues was measured to study whether the differences between the vitamin D 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)_2D_3. The reduced growth inhibitory potential of OCT corresponded with a lower VDR affinity compared to 1,25-(OH)_2D_3, 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 D compounds by cotreatment with tamoxifen was caused by increased VDR binding, we have measured VDR concentration in MC7-7 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, 17B-E_2 had no effect on VDR levels.

**DISCUSSION**

The present data show that the growth inhibitory action of vitamin D analogues and tamoxifen are complementary. In MC7-7 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 D analogues were able to augment the inhibitory effect of tamoxifen, similar as in MC7-7 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 D 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 D compounds.

Despite the promising antiproliferative effects in vitro, the calcemic effects of vitamin D 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)_2D_3 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 17B-E_2-dependent cell line (ZR-75-1) with increased potency compared to 1,25-(OH)_2D_3. In various ER-positive and ER-negative human breast cancer cell lines, OCT was 10 times more potent than 1,25-(OH)_2D_3 (11). In our hands, the potency of OCT was somewhat diminished compared to 1,25-(OH)_2D_3 in both cell lines studied. The reason for this discrepancy is not yet known. CB966 and 1,25-(OH)_2D_3 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 D 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 EC_{50} of 1,25-(OH)_2D_3 and analogues shifted to lower concentrations might be of interest. This shift was more pronounced for 1,25-(OH)_2D_3 and the compounds with an EC_{50} close to 1,25-(OH)_2D_3 (OCT and CB966) than for the compounds with a more favorable EC_{50} (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 EC_{50} of the vitamin D analogues 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.
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)$_2$D$_3$ 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)$_2$D$_3$ 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 17$\beta$-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 17$\beta$-E$_2$. The data obtained with MCF-7 cells clearly indicate an estrogen-independent mechanism of action: (a) the inhibition of 17$\beta$-E$_2$-stimulated growth, expressed in $\mu$g DNA/well, by the vitamin D$_3$ compounds was similar to the inhibition of autonomous growth (maximum and EC$_{50}$); (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 17$\beta$-E$_2$ (33) was not affected; and (d) the expression of the 17$\beta$-E$_2$-regulated pS2 gene was not changed. Moreover, an estrogen-independent mechanism of action is consistent with the fact that 1,25-(OH)$_2$D$_3$ or analogues inhibit breast cancer cell growth, irrespective of the presence of the ER (4, 11). The inhibition of 17$\beta$-E$_2$-induced growth of ZR-75-1 cells seems to contradict an entirely 17$\beta$-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.

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)$_2$D$_3$, which is important to reduce the risk on negative side-effects. Tamoxifen increased the sensitivity to 1,25-(OH)$_2$D$_3$ 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)$_2$D$_3$ and analogues. Thereby, vitamin D$_3$ analogues are interesting candidates for breast cancer therapy.

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