20-epi-Vitamin D₃ Analogues: A Novel Class of Potent Inhibitors of Proliferation and Inducers of Differentiation of Human Breast Cancer Cell Lines¹

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ABSTRACT

We have studied the in vitro biological activities and mechanism of action of 1,25-dihydroxyvitamin D₃ (1,25D₃) and four potent 1,25D₃ analogues (20-epi-22oxa-24a,26a,27α-tri-homo-1,25(OH)₂D₃ (KH 1060); 20-epi-1,25(OH)₂D₃; 1,25(OH)₂-16ene-D₃; and 1,25(OH)₂-16ene-23yne-D₃) on proliferation and differentiation of estrogen receptor-negative (MDA-MB-436, BT-20, SK-BR-3, and MDA-MB-231), estrogen receptor-negative breast cancer cell lines, and on related compounds. Dose-response studies showed that KH 1060 was the most potent analog, because it was able to induce differentiation in all seven breast cancer cell lines (measured by lipid staining) and to suppress more than 50% of clonal proliferation (ED₅₀ at 10⁻⁶ M) in all cell lines, except MDA-MB-436 and BT-20. To explore how these compounds mediated antiproliferative actions, their effects on the cell cycle, and expression of bcl-2 and p53, and on apoptosis were assessed. Five of six cell lines have a mutant p53 gene, whereas MCF-7 has a wild-type p53. Immunohistochemical staining showed that the p53 protein was predominantly localized in the nucleus in each of the breast breast cell lines except for MCF-7, which expressed the protein predominantly in the cytoplasm. After incubation with KH 1060 (3 days; 10⁻⁷ M), expression of bcl-2 protein as determined by immunohistochemical staining was markedly decreased in BT-474, MCF-7, and MDA-MB-231; these same cells were profoundly inhibited in their clonal proliferation and arrested in the G₀/G₁ phase of the cell cycle when cultured with KH 1060. In contrast, BT-20 and MDA-MB-436 cells that were refractory to the antiproliferative effect of KH 1060 (ED₅₀<10⁻⁶ M) had no down-regulation of their bcl-2 expression and no cell cycle changes after exposure to KH 1060. MCF-7 showed morphological changes and DNA fragmentation, indicative of apoptosis after 48 h incubation with KH 1060 (10⁻⁴ M), during which time p53 protein accumulated in the nucleus and decreased in the cytoplasm. In contrast, no apoptosis was detected in three other breast lines (MDA-MB-231, SK-BR-3, and BT-474) that had a mutated p53. In conclusion, the data indicate that KH 1060 is an extremely potent 1,25D₃ analogue inducing differentiation of all six breast cancer line and potentially inhibiting clonal growth of four of them with concomitant decreased bcl-2 and cell cycle arrest at G₀/G₁. Only one (MCF-7) of six breast cancer cell lines underwent apoptosis; these cells have a wild-type p53 that translocated from the cytoplasm to the nucleus during culture with KH 1060, probably allowing p53 to become a functional nuclear transcriptional activator.

INTRODUCTION

Breast cancer remains the most common malignant disease of middle-aged women in the United States. Improvements in hormonal and cytotoxic therapies have not led to either sustained remissions or cures in advanced breast cancer. Although anti-estrogens have pro-

References

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4. To whom requests for reprints should be addressed, at Cedars-Sinai Medical Center/UCLA School of Medicine, 8700 Beverly Blvd., D5033, Los Angeles, CA 90048.

5. The abbreviations used are: ER, estrogen receptor; 1,25D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D₃ receptor; KH 1060, 20-epi-22oxa-24a,26a,27α-tri-homo-1,25(OH)₂D₃; MC 1288, 20-epi-1,25(OH)₂D₃; HM, 1,25(OH)₂-16ene-D₃; V, 1,25(OH)₂-16ene-23yne-D₃.

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were incubated with 9A7, a rat anti-VDR-mAb. Samples were analyzed with a FACSscan flow cytometer. The percentage of positive cells and relative intensity of fluorescence were calculated using the PC LYSIS II software.

MATERIALS AND METHODS

Cell Lines. The breast cancer cell lines (MDA-MB-436, MCF-7, SK-BR-3, BT474, BT20, and MDA-MB-231) were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured according to American Type Culture Collection recommendations in culture flasks with vented filter caps (Costar, Cambridge, MA).

Vitamin D3 Compounds. The vitamin D₃ compounds were dissolved in absolute ethanol at 10⁻³ M as a stock solution, which was stored at −20°C and protected from light. 1,25D₃, HM, and V were synthesized by Hoffmann-LaRoche Inc. (Nutley, NJ). KH 1060 and MC 1288 were synthesized in the Department of Chemical Research, Leo Pharmaceutical Products, Ballerup, Denmark.

Immunofluorescence Staining for Detection of 1,25(OH)₂D₃ Receptors. Breast cancer cell lines were grown on glass chamber slides as monolayers (Lab-Tek; Nunc, Inc. Naperville, IL) at 37°C and 5% CO₂ at a density of 2 × 10⁵ in 3 ml medium. After 3 days of culture, the chambers were removed; the slides were washed with ice-cold Dulbecco's PBS (GIBCO-Life Technologies, Inc. Grand Island, NY) and fixed in ice-cold methanol (10 min) and acetone (10 min). To block nonspecific antibody binding, cells were treated with 10% normal goat serum (Dako, Carpinteria, CA), followed by incubation (60 min at 27°C) with MA1—710, an IgG₂b monoclonal (rat) anti-VDR antibody (Affinity BioReagents, Neshanic Station, NJ; 1:200) with isotype control. Following incubation, cells were washed with Dulbecco's PBS and incubated for 60 min at 27°C with goat anti-rat IgG (H+L) FITC-labeled antibody (1:50; Southern Biotechnology Associates, Inc., Birmingham, AL). Cellular staining was assessed by immunofluorescence microscopy.

Table 1  Expression of VDR, ER, p53, and bcl-2 in breast cancer cell lines

<table>
<thead>
<tr>
<th>Breast cancer cell lines</th>
<th>VDR</th>
<th>ER* staining intensity</th>
<th>p53 mutation status*</th>
<th>bcl-2 staining intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>+</td>
<td>+++</td>
<td>wt</td>
<td>++</td>
</tr>
<tr>
<td>BT-474</td>
<td>+</td>
<td>+</td>
<td>mut</td>
<td>+++</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>+</td>
<td></td>
<td>mut</td>
<td>+</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>+</td>
<td></td>
<td>mut</td>
<td>+</td>
</tr>
<tr>
<td>BT-20</td>
<td>+</td>
<td></td>
<td>mut</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>+</td>
<td></td>
<td>N.I.</td>
<td>+++</td>
</tr>
</tbody>
</table>

* ER data from Ceriani et al. (43) and Dr. Grund (personal communication).

Induction of Differentiation in Breast Cancer Cell Lines. The breast cancer cell lines contain a small fraction of cells that exhibit morphological maturation and differentiation markers associated with milk production; they contain large lipid droplets (32). Breast cancer cell lines were cultured on chamber slides at a density of 2 × 10⁵ in 3 ml medium. At 24 h after seeding at a time when cell adhesion was complete, 1,25D₃, and its analogues were added at 10⁻⁷ M for 3 days without medium change. For lipid visualization, we used a modified Oil Red O in propylene glycol method (33, 34).

Determination of 1,25D₃ Receptors and Cell Cycle Analysis by Flow Cytometry. After trypsinization, extensive washing and fixation for a minimum of 30 min in ice-cold 80% methanol, 10⁶ cells were incubated at 4°C with 1 µg of 9A7, a rat anti-VDR-mAb (Affinity Bioreagents) or normal rat IgG (1:50) for 30 min, followed by FITC-conjugated mouse anti-rat IgG mAb (1:200; Jackson ImmunoResearch, Westgrove, PA) or FITC-conjugated isotype control (Becton Dickinson, Mountain View, CA) for 30 min. Samples were analyzed with a FACSscan flow cytometer (Becton Dickinson). The percentage of positive cells and relative fluorescence intensity were calculated using the PC LYSIS II software.

Cell cycle analysis was performed on breast cancer cells incubated for 48 h with or without KH 1060 at 10⁻⁶ M. The methanol-fixed cells (see above) were incubated for 30 min at 4°C in the dark with a solution of 5 µg/ml propidium iodide, RNase (100 units/ml; Sigma Chemical Co.), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CELLFit program (Becton Dickinson), whereby the S-phase was calculated with an RFit model.

Clonogenic Assay in Soft Agar. Breast cancer cells were cultured in a two-layer soft agar system for 14 days without adding any growth factors, as described previously (31).

Apoptosis. Apoptosis was assessed by analyzing changes in cell morphology and DNA fragmentation after 24, 46, and 72 h of incubation of breast cancer cell lines with KH 1060 at 10⁻⁶ M. Morphologically, cells undergoing apoptosis were identified by intense staining, highly condensed chromatin, fragmented nuclear chromatin, a general decrease in overall cell size, and cellular fragmentation into apoptotic bodies (35). These changes were readily observed in cytospin preparations stained with Diff-Quick Stain Set (Baxter Healthcare Corporation, Miami, FL). Enumeration of apoptotic cells was performed by evaluation of about 300 cells by light microscopy.

The extent of DNA fragmentation was determined by a modification of the method of Sellins and Cohen (36). Positive control for DNA fragmentation was a human T-cell leukemia cell line (CEM) treated for 8 h with anti-CD3 mAb.
Fig. 2. Dose-response effects of vitamin D<sub>3</sub> compounds on clonal proliferation of breast cancer cell lines. Results are expressed as a mean percentage ± SD of control plates containing no vitamin D<sub>3</sub> compounds. Points, means of at least three experiments with triplicate dishes; bars, SD. Control cultures (5 × 10<sup>3</sup>/ml) contained the following colony numbers: MCF-7 (a), 460 ± 13; BT-474 (b), 489 ± 22; MDA-MB-231 (c), 341 ± 17; SK-BR-3 (d), 406 ± 19; BT-20 (e), 55 ± 2; and MDA-MB-436 (f), 262 ± 18.

(OK T3; Ortho Biotech, Raritan, NJ) and MCF7 cells cultured for 6 days without medium change.

To detect apoptotic cells in our samples, we also used the Oncor Apop Tag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) directly detecting digoxigenin-labeled genomic DNA by flow cytometry. The labeling targets of the Apop Tag Kit are new 3'-OH DNA ends generated by DNA fragmentation, typically localized in morphologically identifiable nuclei and apoptotic bodies.

Immunohistochemistry. Immunostaining for bcl-2 and p53 was performed on breast cancer cells that had been grown in culture with and without KH 1060 (10<sup>-7</sup> M) for 3 days, harvested with a disposable cell scraper (Costar, Cambridge, MA), pelleted in saline into cell buttons, and frozen in OCT compound (Miles Diagnostics, Elkhart, IN). Blocks were serially cryostat sectioned. These were air dried and fixed in acetone prior to immunostaining. Intracellular bcl-2 protein was detected with a murine monoclonal anti-bcl-2 antibody (DAKO, Carpinteria, CA). Cryostat sections were stained using a modification of methods described previously (37, 38). The p53 was detected with monoclonal antibodies pAb 1801 (Oncogene Science, Inc., Manhasset, NY).
RESULTS

Expression of 1,25D₃ Receptors in Breast Cancer Cell Lines. Immunofluorescence staining showed that all breast cancer cell lines expressed VDRs that were predominantly localized to the nucleus. Flow cytometry for relative measurements of VDR showed that fluorescence intensity varied between the breast cancer cells, but each had prominent expression of VDR (Fig. 1; Table 1).

Effect of Vitamin D₃ Analogues on Clonal Proliferation of Breast Cancer Cell Lines. Breast cancer cells were cloned in soft agar in the presence of various 1,25D₃ analogues (10⁻¹¹ - 10⁻⁶ M). Data represent three independent experiments with triplicate dishes of each concentration of analogue. Cellular proliferation was determined by counting colonies. Results are expressed as a percentage of control plates containing no 1,25D₃ compounds. Dose-response curves were drawn from data of Fig. 2 and the concentration of analogue achieving a 50% inhibition (ED₅₀) of clonal growth was calculated.

<table>
<thead>
<tr>
<th>Breast cancer cell lines</th>
<th>1,25D₃</th>
<th>KH 1060</th>
<th>MC 1288</th>
<th>HM</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>1.2 × 10⁻⁸</td>
<td>8.2 × 10⁻¹²</td>
<td>1.3 × 10⁻¹⁰</td>
<td>1.0 × 10⁻⁸</td>
<td>5.4 × 10⁻⁹</td>
</tr>
<tr>
<td>BT-474</td>
<td>8.2 × 10⁻⁹</td>
<td>8.1 × 10⁻¹¹</td>
<td>7.4 × 10⁻¹⁰</td>
<td>1.3 × 10⁻⁹</td>
<td>4.4 × 10⁻⁹</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>1.1 × 10⁻⁶</td>
<td>1.0 × 10⁻¹¹</td>
<td>2.2 × 10⁻⁹</td>
<td>N.R.</td>
<td>N.R.</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>8.2 × 10⁻¹¹</td>
<td>8.0 × 10⁻¹²</td>
<td>1.0 × 10⁻¹¹</td>
<td>1.0 × 10⁻⁹</td>
<td>2.4 × 10⁻¹¹</td>
</tr>
<tr>
<td>BT-20</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
<td>N.R.</td>
</tr>
</tbody>
</table>

* N.R., an ED₅₀ was not reached, even at 10⁻⁶ M of the 1,25D₃ compound.

Expression of the p53 protein (40, 41). Incubation with the primary antibody (30 min) was followed by biotinylated universal anti-immunoglobin antibodies (Shandon-Lipshaw, Pittsburgh, PA) and peroxidase-conjugated streptavidin (Shandon-Lipshaw). Antibody localization for bcl-2 and p53 was performed with 3,3'-diaminobenzene hydrochloride (5 mg/10 ml; Sigma), to which 0.03% hydrogen peroxide was added just before use. Slides were counterstained with methyl green and mounted with permount. Positive controls were included with each analysis and consisted of sections of a known p53-overexpressing breast carcinoma, whereas negative controls consisted of substitution of the p53-specific antibody with isotype-specific monoclonal antibody. Additional negative controls consisted of SKOV3 cells, a human ovarian carcinoma line that does not express p53 (42). Staining was evaluated by two investigators in a double-blinded manner.

Induction of Differentiation of Breast Cancer Cell Lines. The analysis of differentiation of breast cancer cells was determined by the expression of lipid in these cells after exposure to 1,25D₃ and 1,25D₃ analogues (10⁻⁷ M) for 3 days (Fig. 3). Approximately 10% of control cells expressed lipid. Almost all of the analogues induced lipid expression in each of the breast cancer cell lines. The most potent inducer of differentiation was KH 1060, with between 30 and 90% of the cells becoming lipid positive in the various lines. The MC 1288 and HM were the next most potent compounds, and analogue V was, in general, only slightly more potent than 1,25D₃. Interestingly, the 1,25D₃ compounds and in particular KH 1060 induced differentiation of BT20 and MDA-MB-436, which were refractory to inhibition of proliferation by VD₃ analogues. For example, 70 to 80% of these cells became lipid positive in the presence of KH 1060 (Figs. 3, e and f). In contrast, these cells were resistant to the antiproliferative affects of the analogues (Fig. 2, e and f). This suggests a dissociation between induction of differentiation and inhibition of proliferation of these breast cancer cell lines.

Cell Cycle Analysis. Effect of KH 1060 on the cell cycle of breast cancer cells was determined by studying four sensitive cell lines (MCF-7, BT 474, MDA-MB-231, and SK-BR-3) and two resistant lines (BT 20 and MDA-MB-436) to the antiproliferative effects of the KH 1060. MCF-7, BT 474, MDA-MB-231, and SK-BR-3 had a significant increase in the numbers of cells in the G₀/G₁ phase of the cell cycle after exposure to KH 1060 (10⁻⁶ M) for 48 h (P < 0.05; Table 3). On the other hand, MDA-MB-436 and BT 20, which were resistant to clonal inhibition by the 1,25D₃ analogues including KH 1060 (Fig. 21), showed no changes in the cell cycle after incubation with KH 1060 (10⁻⁶ M) for 48 h (Tables 3 and 4).

Induction of Apoptosis by KH 1060 in Breast Cancer Cell Lines. The strong antiproliferative effects of KH 1060 on breast cancer cells in vitro may be caused by induction of apoptosis. To test this hypothesis, we examined the cell morphology and measured DNA fragmentation as markers for apoptosis. Neither morphological changes nor DNA fragmentation was detected in BT474, SK-3, and MDA-MB-231 after 3, 24, 48, and 72 h of cultivation with KH 1060 (10⁻⁶ M). In contrast, a mean 42 ± 8% of MCF-7 breast cancer cells showed apoptotic morphological changes and DNA fragmentation after 48 h of incubation with KH 1060 (10⁻⁶ M; Figs. 4 and 5a). As shown in Fig. 5b, a pronounced increase in DNA strand breaks was observed in MCF-7 breast cancer cells undergoing an apoptotic response to KH 1060.

Decreased Levels of bcl-2 Induced by Exposure of Breast Cancer Cell Lines to KH 1060. Double-blinded analysis by immunohistochemistry showed that MCF-7, BT 474, and MDA-MB-436 cell lines had strong cytoplasmic staining (2-3+) intensity for bcl-2; MDA-MB-231 and BT 20 stained weakly (1+), and SK-BR-3 was negative for bcl-2 (Table 1; Fig. 6). After treatment with KH 1060 (10⁻⁷ M for 3 days), the intensity of staining for bcl-2 decreased in MCF-7, BT474, MDA-MB-231, whereas little or no change in expression of bcl-2 was observed in BT-20, MDA-MB-436, and SK-BR-3 cells (Fig. 6).
Nuclear Migration of p53 in MCF-7 Breast Cancer Cells after Exposure to KH 1060. The p53 protein was present predominantly in the nucleus in all of the cell lines except for MCF-7, which had immunohistochemical staining for p53 mainly in the cytoplasm (Table 1). Interestingly, after exposure to KH 1060 (10^{-7} M for 3 days), MCF-7 showed a shift in localization of p53 from the cytoplasm to the nucleus (Fig. 7).

**DISCUSSION**

This study had two principal aims: (a) to identify vitamin D$_3$ analogues that potently inhibit clonal proliferation of breast cancer cells; and (b) to develop a greater understanding concerning the mechanism of action of these compounds by investigating their effects on a variety of breast cancer cell lines having different biological profiles. These studies are important because novel nontoxic therapies are needed for the breast cancer patient who has been placed in remission but has a high likelihood of relapse and/or has a high risk of developing contralateral breast cancer. The compounds studied here were selected because they have been shown to be very potent in the inhibition of clonal proliferation of a variety of myeloid leukemic cell (20, 22, 27, 29–31).

KH 1060 was the most active analogue in the myeloid leukemia models and also in the present studies had the greatest efficacy as an inhibitor of clonal growth of the breast cancer cell lines. Remarkably, this compound was up to 4000-fold more potent than the physiologically active 1,25D$_3$. This analogue belongs to a new class of vitamin D$_3$ analogues that have an alteration of the
stereochemistry at carbon 20 on the side chain, resulting in 20-epi-vitamin D$_3$ analogues. The other 20-epi-vitamin D$_3$ analogue (MC 1288) examined in this study was also a very potent inhibitor of clonal proliferation of breast cancer cell lines (Table 2; Fig. 3).

The reasons for the remarkable potency of the 20-epi family of 1,25D$_3$ compounds are presently unknown. The 20-epi analogues differ markedly from 1,25D$_3$ in their conformational distribution (45). This means that the side chain of the 20-epi-vitamin D$_3$ analogues is directed to the left, where it is directed to the right in the "normal" isomers. This can induce significant changes in the conformation of the receptor upon ligand binding, thereby inducing differences in the biological selectivities of various compounds. Our previous transactivation studies using a reporter gene containing an osteocalcin vitamin D$_3$ response element suggested that the difference between MC 1288 and 1,25D$_3$ cannot be ascribed to differential abilities either to enter the cells or to bind to VDRs, because both 1,25D$_3$ and MC 1288 increased nearly identically the reporter gene activities in a dose-response manner (31). However, several other studies have suggested that the 20-epi analogues more efficiently transactivated a reporter gene having a vitamin D$_3$ response element and more efficiently enhance homo- and heterodimerization of VDR, with VDR and RXR as compared to normal 1,25D$_3$ isomers (46, 47). Recently, evidence has accumulated to suggest that some effects of 1,25D$_3$ analogues may be mediated independently of the 1,25D$_3$ nuclear receptors. For example, several 1,25D$_3$ analogues have been found to cause intracellular Ca$^{2+}$ fluxes through a nongenomic mechanism that is independent of the classical pathway of receptor-mediated activity (48).

Table 3 Cell cycle analysis of breast cancer cells cultured with KH 1060
Each point represents a mean of at least three independent experiments. The cells were cultured with KH 1060 at $10^{-6}$ M for 2 days before cell cycle analysis. Control cells represent those not exposed to KH 1060.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>G$_0$/G$_1$-phase</th>
<th>S-phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>KH 1060 ($10^{-6}$ M)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>53.4 ± 8</td>
<td>74.9 ± 2.9*</td>
</tr>
<tr>
<td>BT474</td>
<td>50.2 ± 9</td>
<td>61.7 ± 5.4*</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>55.6 ± 0.1</td>
<td>64.4 ± 3.2*</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>47.5 ± 18</td>
<td>75 ± 4*</td>
</tr>
<tr>
<td>BT20</td>
<td>51.4 ± 10</td>
<td>53.8 ± 6</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>37.5 ± 3.8</td>
<td>34.6 ± 3</td>
</tr>
</tbody>
</table>

* $P < 0.05$ as determined by Student's $t$ test.

Since previous data from our group suggested that the 20-epi-vitamin D$_3$ analogue MC 1288 mediates its effects through the classical VDR pathway (31, 49), the increased potencies of these analogues may be associated with postreceptor binding effects (50).

The breast cancer cell lines in our study varied in their sensitivities to the clonal inhibitory effects of the various 1,25D$_3$ compounds. The...
most sensitive lines, MCF-7, SK-BR-3, and BT-474, were inhibited by all of the analogues. In contrast, MDA-MB-231 exhibited a slight stimulation of clonal proliferation to 1,25D$_3$, HM, and V over a wide range of concentrations but showed a good response to the antiproliferative effects of the 20-epi vitamin D$_3$ compounds (KH 1060 and MC 1288). This suggests the importance of examining these 20-epi-D$_3$ analogues in clinical trials and further suggests that they may have a different mode of action as compared to the other analogues. Furthermore, MDA-MB-436 and BT-20 were resistant to the antiproliferative effect of all of the 1,25D$_3$ compounds used in this study, suggesting that preclinical testing of the tumors in vitro may be helpful in selection of patients for clinical trials with these analogues.

The variation in the responsiveness of the breast cancer cell lines to the clonal inhibitory effects of the 1,25D$_3$ analogues provides an interesting model system. This variation in sensitivity cannot be explained by variation in expression of immunodetectable VDR or ER. Indeed, three of the lines (MDA-MB-231, BT-20, and MDA-MB-436) that were nearly refractory to the growth-inhibitory effects of VD$_3$ had prominent expression of VDR (Fig. 1), suggesting that VDR expression may be necessary but not sufficient for responsiveness to 1,25D$_3$. One study suggested that the antiproliferative effects of 1,25D$_3$ results from its antagonism to the action of estrogen (51). However, our results cannot support this hypothesis, because three of the four cell lines that did not express ERs were refractory to the growth-inhibitory effects of 1,25D$_3$.

Analysis of differentiation of breast cancer cell lines, as measured by expression of lipid markers, showed that the 1,25D$_3$ compounds induced differentiation of each of the breast cancer cell lines. The most potent inducer of differentiation was KH 1060. Interestingly, KH 1060 caused the differentiation of 69–89% of BT20 and MDA-MB-436 cells; in contrast, these cell lines were resistant to the antiproliferative effects of this analogue in clonogenic assays without any changes in their cell cycle. Also, MCF-7 cells had the weakest induction of differentiation by KH 1060 and the other 1,25D$_3$ compounds; nevertheless, this cell line was very sensitive to clonal inhibition by 1,25D$_3$ and to arrest in the G$_0$/G$_1$ cell cycle after culture with KH 1060. Taken together, these results suggest that breast cancer cells can undergo at least partial phenotypic differentiation and still actively proliferate. The data suggest that regulation of proliferation and differentiation by 1,25D$_3$ compounds may occur through different VDR-mediated pathways. These findings are in contrast to those of acute myeloid leukemia cells in which cessation of their proliferation and induction of their differentiation by the 1,25D$_3$ analogues is closely linked (25, 31, 52, 53). Further studies are required for the identification and study of the genes that are directly modulated by these novel 1,25D$_3$ analogues and are necessary for the cessation of proliferation and induction of differentiation of breast cancer cells.

Therapeutic agents can induce tumor regression through inhibition of proliferation, activation of programmed cell death (apoptosis), or both. Apoptosis is an active process and depends on expression of a specific set of genes including p53 and bcl-2 (54–56). To attempt to understand the mechanisms involved in the antiproliferative actions of the 1,25D$_3$ analogues, the effects of KH 1060 on expression of bcl-2 and p53 and development of apoptosis were assessed in the breast cancer cell lines having different sensitivities to the antiproliferative action of this analogue. The bcl-2 protein, which is overproduced in many types of human tumors, suppresses apoptosis induced by a wide variety of stimuli, including chemotherapeutic drugs and γ-irradiation (57–60). After incubation of the breast cancer cell lines with KH 1060, expression of the bcl-2 protein was markedly decreased in BT-474, MCF-7, and MDA-MB-231 cell lines. The same cell lines were strongly inhibited in their clonal proliferation after treatment with KH 1060. Furthermore, the BT-20 and MDA-MB-436 cells, which were refractory to the antiproliferative effect of KH 1060, had down-regulation of their bcl-2 after exposure to KH 1060 (Table 4), and one cell line (SK-BR-3) that expressed negligible levels of bcl-2 before and after exposure to KH 1060 was very sensitive to the growth inhibitory effects of the analogue. Since all cell lines with a decreased level of expression of bcl-2 after exposure to KH 1060 or with negligible constitutive expression of bcl-2 were markedly inhibited in their clonal growth by the 1,25D$_3$

![Fig. 6. The effect of KH 1060 on expression of bcl-2 gene in breast cancer cell lines as measured by intensity of staining as determined by immunohistochemistry. Data represent means of at least three independent experiments, without (−) and with (+) exposure of the cells to KH 1060 (10$^{-7}$ M for 3 days); bars, SD.](image)

![Fig. 7. The effect of KH 1060 on expression of p53 in the nucleus of breast cancer cells as measured by p53 immunohistochemistry. Results are expressed as a percentage of breast cancer cells that had immunostaining for p53 in the nucleus. Data represent means of at least three independent experiments, without (−) and with (+) exposure to KH 1060 (10$^{-7}$ M) for 3 days; bars, SD.](image)
analogues, we believe that alterations of the levels of bcl-2 are directly or indirectly involved in the antiproliferative effects of the 1,25D₃ analogues.

Wild-type p53, a tumor suppressor protein, acts as a guardian for repair of DNA damage by preventing progression of the cell cycle from G1 to S; this is mediated by p53 inducing the expression of p21, which is involved in repair of DNA damage by preventing progression of the cell cycle. In addition, p53 has been suggested as a key regulator of apoptosis (63–65). The critical role played by abnormal p53 in human breast cancer is well established. About 30 to 50% of breast cancers have a mutant p53 gene, and additional breast cancer samples have alterations in expression of p53 (66, 67). Patients with p53 mutations in inflammatory breast carcinoma have a poor prognosis (68); those breast cancer cell lines with mutant p53 have significantly higher levels of metastasis to the lung when transplanted s.c. into animals as compared to those having no p53 mutations (69). Moll et al. (70) have found that cells from about 30% of breast cancers have a nonfunctional wild-type p53 because the protein is inappropriately located in the cytoplasm of tumor cells. Five of the six breast cancer cell lines used in our study have a mutation of the p53 gene; MCF-7 has wild-type p53. The mutated p53 protein was detected predominantly in the nucleus of these cell lines, except for MCF-7 which had p53 mainly localized in the cytoplasm. Because p53 is a nuclear transcription factor, cytoplasmic p53 probably is inactive; therefore, each of the cell lines used in this study had a nonfunctional p53. Furthermore, apoptosis was studied in MDA-MB-231, SK-BR-3, BT-474, and MCF-7 cells after different durations of exposure to KH 1060; no apoptosis was detected in BT-474, SK-BR-3, and MDA-MB-231. Nevertheless, KH 1060 resulted in 80–100% inhibition of their clonal proliferation and arrest in G₁/G₀/G₂, suggesting that the prominent antimitotic effect of this analogue in these breast cancer cell lines was independent of apoptosis. In contrast, apoptosis occurred in MCF-7 cells after 48 h of incubation with KH 1060. Interestingly, during exposure to KH 1060, the wild-type p53 protein translocated from the cytoplasm to the nucleus of the MCF-7 cells, presumably resulting in a functionally active protein that could then facilitate the induction of apoptosis.

The factors regulating intracellular transport of p53 are unknown. When murine erythroleukemic cells, transfected with a temperature-sensitive mutant p53, are exposed to the differentiating inducing agent DMSO, the p53 changes from mutant to wild-type conformation. This is accompanied by translocation of the p53 protein from the cytoplasm to the nucleus; and the cells, rather than differentiating terminally, are induced to undergo apoptosis (71). These findings suggest that activity of p53 can be influenced through exposure to differentiating agents. Perhaps one of the mechanisms by which KH 1060 induces apoptosis in MCF-7 is to induce the transport of p53 from the cytoplasm to the nucleus, where it can assume its function as a transcriptional activator and as a trigger of apoptosis.

Table 4 summarizes our data concerning the effects of KH 1060 on breast cancer cell lines in vitro; we have identified a group of 1,25D₃ analogues (especially KH 1060) with potent antiproliferative and differentiative effects on breast cancer cells in vitro. These interesting compounds should be studied in vivo in appropriate breast and other cancer model systems.

### REFERENCES


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20-epi-Vitamin D₃ Analogues: A Novel Class of Potent Inhibitors of Proliferation and Inducers of Differentiation of Human Breast Cancer Cell Lines

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