Antiestrogen Potentiation of Antiproliferative Effects of Vitamin D₃ Analogues in Breast Cancer Cells

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ABSTRACT

[³H]thymidine incorporation and DNA content were used to investigate the antiproliferative effects of 1,25(OH)₂D₃ and four analogues [16-ene-1,25(OH)₂D₃ (16-ene); 16-ene,23-yne-1,25(OH)₂D₃; EB1089; and 22-oxa-1,25(OH)₂D₃] on MCF-7, BT-474, and MDA-MB-453 breast cancer cell lines. 1,25(OH)₂D₃ and the analogues elicited a biphasic response from MCF-7 and BT-474 estrogen receptor (ER)-positive cells, in the presence of estradiol (E₂), with lower doses (between 10⁻¹² and 10⁻¹⁰ M) tending to stimulate proliferation and higher doses (between 10⁻⁸ and 10⁻⁶ M) inhibiting proliferation by as much as 65%. In the absence of E₂, the stimulatory effect was abrogated. Proliferation of MDA-MB-453, estrogen receptor-negative (ER⁻) cells, was stimulated by these compounds only at 10⁻¹⁰ M, and inhibited by all higher doses, by as much as 83%. All three cell lines were shown to be vitamin D receptor (VDR) positive, and 1,25(OH)₂D₃ and all four analogues bound to the VDR with high affinities in each cell line. The antiestrogen ICI 164,384 inhibited the proliferation of all three cell lines. ICI 164,384 at 10⁻⁸ M in combination with 1,25(OH)₂D₃ or EB1089 converted biphasic response of the ER⁺ cells to one resembling the response of the ER⁻ cells, by eliminating the stimulatory response elicited by 1,25(OH)₂D₃ at low doses and enhancing the antiproliferative effects of higher doses by as much as 1000-fold. These data are consistent with the hypothesis that E₂ in the ER⁺ cells blocks the antiproliferative effects of the analogues and suggest the potential usefulness of combined antiestrogen and 1,25(OH)₂D₃ analogues in ER⁺ breast tumors, whereas 1,25(OH)₂D₃ analogues alone might suffice in ER⁻ breast tumors.

INTRODUCTION

Breast cancer is one of the leading causes of mortality among women in the United States, despite intense efforts to develop better chemo- or hormonal therapies. The probability of death at 5 years after diagnosis is reduced with tamoxifen treatment in women more than 50 years of age, and some chemotherapeutic drugs also confer a survival advantage in younger patients (1); however, side effects of most cancer therapies are substantial, and many tumors that initially respond to tamoxifen will eventually become resistant.

1,25(OH)₂D₃, the most biologically active form of vitamin D₃, plays a critical role in the regulation of calcium through binding to its nuclear receptor in bone, intestine, and kidney (2). It has been demonstrated that the VDR is expressed widely in a large variety of cell types, including keratinocytes and hematopoietic cells; 1,25(OH)₂D₃ is involved in the regulation of proliferation and differentiation of these cells (3).

Since the first demonstration of a specific receptor for 1,25(OH)₂D₃ in breast cancer cells (4), subsequent studies have revealed the presence of the VDR in a high percentage of breast carcinomas (5). Patients with VDR⁺ tumors tend to experience significantly longer disease-free survival than those with VDR⁻ tumors (6). Recent experimental evidence suggests a role for 1,25(OH)₂D₃ in the regulation of growth of breast cancer cells (7-10). These nonclassical effects of 1,25(OH)₂D₃ offer promise for its use as an antiproliferative treatment. The doses of 1,25(OH)₂D₃ necessary to achieve tumor suppression, however, are likely to result in the development of hypercalcemia.

1,25(OH)₂D₃ analogues with less ability to induce hypercalcemia have been developed in an effort to dissociate the effects on proliferation and differentiation from those on calcium metabolism by bone and intestine. Both in vitro antiproliferative effects and in vivo suppression of tumor growth have been demonstrated for some of the analogues used in this study (8, 11, 12). This evidence, combined with the high percentage of VDR expression in human breast tumors (5), indicates a potential role for the 1,25(OH)₂D₃ analogues in the treatment of breast cancer.

E₂ is critically important for the growth of normal breast cells, as well as many breast cancer cells, and antiestrogens are routinely utilized for the treatment of breast cancer. However, ER⁺ tumors would not be expected to respond to antiestrogen treatment, and some ER⁻ tumors lose expression of ER during treatment. Dual therapy, e.g., antiestrogens combined with 1,25(OH)₂D₃ analogues, could have enhanced efficacy using lower doses of each and could be effective against both ER⁺ and ER⁻ breast cancer cells. Recent studies have examined the effects of 1,25(OH)₂D₃ and some of the analogues on levels of ER expression. One such study demonstrated that 1,25(OH)₂D₃ and its analogue EB1089 down regulate ER expression in MCF-7 cells (11). Thus, one potential mechanism for the antiproliferative action of 1,25(OH)₂D₃ may be a reduction in ER expression, although this mechanism has been questioned by a second study (10). The development of well-tolerated therapy that inhibits or reverses the proliferation of all tumors, regardless of ER expression, is critically important.

In the current study, we compared four analogues to 1,25(OH)₂D₃ for their ability to inhibit the in vitro proliferation of breast cancer cell lines with varying levels of ER expression. In addition, we determined the levels of VDR mRNA and protein expression in these breast cancer cell lines, and the affinity of the analogues for the VDR. We also investigated the potential for the antiestrogen ICI 164,384 to synergize with 1,25(OH)₂D₃, because dual treatment with 1,25(OH)₂D₃ analogues and ICI 164,384 may provide a more beneficial effect on breast cancer, allowing the use of lower doses of each compound, and controlling both ER⁺ and ER⁻ cells.

MATERIALS AND METHODS

Cell Culture. MCF-7 (ER⁺/+), BT474 (ER⁺/+), and MDA-MB-453 (ER⁻) breast cancer cell lines were obtained from Dr. C. Benz, University of California, San Francisco, California. Cells were maintained in DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% fetal bovine serum (basal medium), at 37°C in a humidified atmosphere of 5% CO₂ in air. Cell lines were passaged twice weekly. Insulin/transferrin/selenium (ITS+), for use in serum-free assays, was obtained from Collaborative Bio-
medical products (Bedford, MA) and added to DMEM at a concentration of 1%.

**Compounds.** 1,25(OH)$_2$D$_3$, 16-ene, and 16-ene,23-yne were obtained from Dr. Milan Usubkovic, Hoffmann-La Roche, Inc. (Nutley, NJ). EB1089 was obtained from Dr. Lise Binderup, Leo Pharmaceutical Products (Ballerup, Denmark). OCT was obtained from Dr. Yasuo Nishii, Chugai Pharmaceuticals Co., Ltd. (Shizuoka, Japan). All analogues used in the current study were synthesized as modifications of the C17 side chain of 1,25(OH)$_2$D$_3$. Fig. 1 depicts the chemical structure of 1,25(OH)$_2$D$_3$ and the four analogues. 26,27-
$[^{3}H]$1,25(OH)$_2$D$_3$ (177 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). ICI 164,384 was obtained from Dr. Alan Wakeling, Zeneca Pharmaceuticals, Inc. (Macclesfield, England). For assays, compounds were dissolved in absolute ethanol at 1 x 10$^{-4}$ M, and dilutions were made in absolute ethanol. The maximum concentration of ethanol in culture (1%) did not influence growth.

**Quantitation of ER.** ER levels in the three cell lines were determined using a commercially available monoclonal ER-EIA kit (Abbott Laboratories, Abbott Park, IL). The recommended kit protocol was followed, with the exception that 0.4 $\mu$M KC$_2$H$_2$O$_4$ was added to the homogenization buffer to facilitate extraction of occupied receptor.

**Proliferation Assay.** The effects of the 1,25(OH)$_2$D$_3$ analogues and the antiestrogen ICI 164,384 on the proliferation of the breast cancer cell lines in vitro were assessed by $[^{3}H]$thymidine incorporation as well as DNA content (see below). Cells were washed twice in basal medium and then were seeded into 24-well dishes at a density of 3 x 10$^4$ cells/well for MCF-7, 5 x 10$^4$ cells/well for BT474, and 2 x 10$^5$ cells/well for MDA-MB-453, in basal medium (described above). These seeding densities were determined in preliminary experiments to result in optimal growth of each cell line. After 48 h, cells were changed to phenol red-free, serum-free basal medium containing 1% insulin-transferrin-selenium. After an additional 24 h, compounds or vehicle (1.0% ethanol) were added (day 0). Medium and test compounds were changed after 2 additional days of incubation. Wells were pulsed with 1 $\mu$Ci/well $[^{3}H]$thymidine, 177 Ci/mmol (Amersham) 16 h prior to harvest at 3 days of incubation. The incorporated radioactivity in the cells was measured by liquid scintillation counting on a LS5000TA $\beta$-counter (Beckman Instruments) after trichloroacetic acid precipitation and NaOH solubilization.

**DNA Assay.** Cells were plated and treated as for proliferation, then removed from wells by scraping in 1 ml of a buffer containing 2 M NaCl, 0.05 $\mu$M Na$_2$PO$_4$, and 2 mM EDTA, pH 7.4 (Hoechst buffer). Samples were then sonicated for 12 s with a Pellet Pestle (Kontes, Vineland, NJ). Samples (50–1000 $\mu$l) were used for DNA determinations by incubating with Hoechst dye 33258 (Calbiochem-Behring Corp., La Jolla, CA), according to the method of LaBarca and Paigen (13). Standards of calf thymus DNA (0–5 $\mu$g) were used, and samples and standards were analyzed on a 650–400 fluorimeter (Perkin-Elmer Corp., Norwalk, CT).

**Quantitation of VDR.** Analysis of 1,25(OH)$_2$D$_3$ receptors in breast cancer cell extracts was performed as described by Halloran and De Luca (14). Briefly, cells were grown to confluence, washed in basal medium, and then washed once and resuspended in 4 ml TKEDM buffer (50 mM Tris-HCl, 300 mM Na$_2$CO$_3$, 1.5 mM EDTA, 5 mM DTT, and 10 mM sodium molybdate, pH 7.4). Cells were sonicated in TKEDM buffer on ice for three 15-second intervals at the maximum setting, using an Ultrasonics W-380 (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) and then were centrifuged at 80,000 x g for 1 h. The cytosol (supernatant) was used as the VDR source. Activity was measured by combining 380 $\mu$l cytosol (containing approximately 800 $\mu$g protein, as measured by the Bradford protein assay) in TKEDM, 5 x 10$^3$ cpm $[^{3}H]$1,25(OH)$_2$D$_3$ (0.065 nm) in 4 $\mu$l ethanol, and increasing amounts of nonradioactive 1,25(OH)$_2$D$_3$ or analogue (0.05–100 nm) in 16 $\mu$l ethanol. Nonspecific binding (binding obtained in the presence of 100 nm nonlabeled 1,25(OH)$_2$D$_3$ or analogue) was subtracted from the total binding to determine specific binding. Sample tubes in triplicate were incubated on a shaker for 6 h at 0–4°C, conditions found to provide maximum binding in preliminary experiments. Unbound 1,25(OH)$_2$D$_3$ was removed by the addition of 100 $\mu$l of 0.15% dextran T70 and 1.5% charcoal in TKEDM buffer. Charcoal was removed by centrifugation at 1500 rpm for 5 min, after 10 min incubation at 4°C. Radioactivity in the supernatant was determined by liquid scintillation counting. Analysis of the data was performed by the method of Scatchard (15), using computer-assisted linear regression, and shown in displacement curves with dissociation constants presented in table form.

**RNA Isolation and Hybridization.** RNA was extracted from cells, using RNA STAT-60™, (TEL-TEST “B” Inc., Friendswood, TX). Briefly, total RNA was extracted with RNA STAT-60 followed by extraction with chloroform, and then the aqueous phase was precipitated with 100% isopropanol and washed with 75% ethanol and 100% ethanol. Samples of 25 $\mu$g were fractionated on a 1% agarose-formaldehyde gel and transblotted onto a Hybond N+ nylon membrane (Amersham) with 50 $\mu$m NaOH. Prehybridization was performed at 42°C for 1 h and followed by hybridization at 42°C overnight with a $[^{32}P]$:random-prime-labeled cDNA probe for human 1,25(OH)$_2$D$_3$ receptor (courtesy of Dr. J. Wesley Pike, Ligand Pharmaceuticals, San Diego, CA). Low-stringency washes and subsequent high-stringency washes were performed two times each with solutions containing 0.2X SSC (0.15 M NaCl, 0.015 M Na$_2$PO$_4$, citrate-2H$_2$O).0% SDS or 0.2X SSC/0.5% SDS, respectively, at 60°C for 30 min. Blots were then exposed to autoradiograph film (Kodak) and message levels quantitated by the NIH Image 1.52 program. VDR mRNA levels were normalized by reprobing each blot for 18 S RNA.

**Statistical Analysis.** Data were analyzed by appropriate methods in Microsoft Excel (Microsoft Corp.). The dose-response effects of vitamin D analogues and ICI 164,384 were tested for significance by ANOVA. Individual points within experiments were compared by Student's two-tailed t test.

**RESULTS**

**ER Expression in MCF-7, BT-474, and MDA-MB-453 Cells.** Levels for each of the three cell lines were 88 fmol/mg cytosolic protein (MCF-7), 24 fmol/mg (BT-474), and 5 fmol/mg (MDA-MB-453). Values less than 15 fmol/mg are considered negative by the manufacturers of the assay kit. Values obtained for duplicate samples were within 0.025 absorbance units of the mean for results less than 0.2 and were within 12% of the mean for results greater than 0.2, as required by the kit.

**Effects of 1,25(OH)$_2$D$_3$ Compounds on the Proliferation of MCF-7, BT-474, and MDA-MB-453 Cells in Vitro.** As measured by $[^{3}H]$thymidine incorporation and DNA content, all three cell lines were able to proliferate in serum-free, phenol red-free medium (data not shown). Because breast cancer cells, as well as normal breast tissue, are profoundly influenced in growth and development by E$_2$, and MCF-7 and BT-474 cells are ER$^+$ and E$_2$-responsive, the effects of 1,25(OH)$_2$D$_3$ and the analogues were investigated in the presence of 10$^{-10}$ M E$_2$-stimulated proliferation of these two cell lines. The results of $[^{3}H]$thymidine incorporation are shown in Fig. 2. The data for DNA content are comparable and are not shown. As depicted in

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4 Unpublished data.
Fig. 2a, 1,25(OH)_{2}D_{3} and the four synthetic analogues tended to stimulate proliferation of MCF-7 and BT-474 at low doses (between \(10^{-12}\) and \(10^{-10}\) M), whereas they inhibited proliferation at higher doses (between \(10^{-9}\) and \(10^{-6}\) M). The exception was EB1089 on BT-474 cells, which was not stimulatory at any dose in this experiment, although mild stimulation of this cell line by EB1089 at low doses was seen in other experiments. The stimulation of proliferation, as analyzed by ANOVA, was statistically significant for BT-474 cells treated with 16-ene (0.0003), 16-ene,23-yne (0.0018), and OCT (0.0015) and for MCF-7 cells treated with all compounds except 16-ene,23-yne [1,25(OH)_{2}D_{3} 0.0011, 16-ene 0.0013, EB1089 0.0002, and OCT 3.53E-05]. MDA-MB-453 cells are ER~ and E2-unresponsive. Therefore, E2 was not added during assays of this cell line. All five compounds tended to stimulate the proliferation of MDA-MB-453 cells at \(10^{-12}\) M in this experiment (although 1,25(OH)_{2}D_{3} was the only compound that was statistically significant over control, when analyzed by Student’s two-tailed t test) and then to inhibit their proliferation at \(10^{-11}\) M and above. Certain analogues proved to be 10- to 1000-fold more potent than 1,25(OH)_{2}D_{3} in inhibiting proliferation. EB1089 was the most effective analogue in inhibiting the proliferation of BT-474 and MDA-MB-453 cells (1000-fold and 10-fold more potent than 1,25(OH)_{2}D_{3}, respectively), whereas 16-ene,23-yne was most effective on MCF-7 cells [100-fold more potent than 1,25(OH)_{2}D_{3}]. OCT and 16-ene tended to be less effective than 1,25(OH)_{2}D_{3}. The ER~ MDA-MB-453 cells were the most profoundly inhibited by all compounds, with inhibition of proliferation as much as 83% by EB1089 at \(10^{-6}\) M. In a subsequent experiment, shown in Fig. 2b, when MCF-7 and MDA-MB-453 cells were incubated with \(10^{-12}\) to \(10^{-6}\) M 1,25(OH)_{2}D_{3} or EB1089 in the absence of E_2 there was no change in the profile of the response of MDA-MB-453 cells; however, the stimulation of proliferation of MCF-7 cells by physiological doses of these compounds was abrogated (analyzed by ANOVA; \(P < 0.0007\)).

**VDR mRNA and Protein Expression.** Fig. 3 illustrates the mRNA levels of the VDR for each of the three cell lines. The mRNA levels were approximately equivalent for MCF-7 and BT-474, but were about 20% lower for MDA-MB-453 (corrected by 18S RNA) as quantitated by densitometry. All three cell lines were then examined for expression of the VDR, and for the affinity of 1,25(OH)_{2}D_{3} and the analogues (Fig. 4). MCF-7 and BT-474 cells had comparable VDR concentrations (64 ± 15 and 76 ± 14 fmol/mg protein, respectively), whereas MDA-MB-453 cells had levels 2.7- to 3.2-fold higher, 203 ± 24 fmol/mg protein. The affinity of the receptor for 1,25(OH)_{2}D_{3} was comparable in all three cell lines, 0.3-0.9 nm (Table 1). All analogues bound to the VDR with an affinity within one order of magnitude of that for 1,25(OH)_{2}D_{3}. The rank order in BT-474 was EB1089 > 16-ene,23-yne > 1,25(OH)_{2}D_{3} > OCT = 16-ene, in MCF-7 was 16-ene,23-yne = 16-ene > EB1089 = 1,25(OH)_{2}D_{3} > OCT, and in MDA-MB-453 was 1,25(OH)_{2}D_{3} > OCT ≈ 16-ene,23-yne.
Fig. 3. VDR mRNA levels in breast cancer cells. VDR mRNA levels were measured by Northern blot analysis, and normalized to 18 S RNA.

*yne > EB1089 > 16-ene. This rank order of affinity in general corresponded to the rank order of potency by the antiproliferative actions of the analogues for BT-474 and MCF-7, although not for MDA-MB-453.

**Effects of ICI 164,384 Alone and in Combination with 1,25(OH)2D3 on the Proliferation of MCF-7, BT-474, and MDA-MB-453 Cells.** Proliferation of MCF-7 and BT-474 cells was stimulated by almost 20% by 10^-10 M ICI 164,384, then inhibited by 10^-8 M and above. Surprisingly, proliferation of the ER MDA-MB-453 cells was also inhibited by ICI 164,384 (Fig. 5). Proliferation of MDA-MB-453 was consistently (three experiments) inhibited to a greater degree by 10^-10 M ICI 164,384 than by 10^-8 M (P < 0.01 for control versus 10^-10 M and 10^-12 versus 10^-9 M, but not for control versus 10^-9 M).

ICI 164,384 at 10^-8 M, when added with the full range of 10^-12 to 10^-6 M 1,25(OH)2D3 to MCF-7 or BT-474 cells, eliminated the proliferative effect of low doses of 1,25(OH)2D3, converting the biphasic response of the ER+ cells to one resembling the ER- MDA-MB-453 response (Fig. 6). The proliferative effect of low doses of 1,25(OH)2D3 was overcome, except for the stimulatory response of MCF-7 to 10^-12 M 1,25(OH)2D3, and the antiproliferative response was enhanced markedly. Inhibition of proliferation of MDA-MB-453 cells by 1,25(OH)2D3 was also increased by the addition of 10^-8 M ICI 164,384 (analyzed by ANOVA; P < 0.0002) but not to the same extent as seen in the ER+ cell lines. The data for DNA content for both studies involving ICI 164,384 are comparable and are not shown.

**DISCUSSION**

After the demonstration that the VDR is expressed in breast cancer cells (4) and the development of potent nonhypercalcemic 1,25(OH)2D3 analogues, subsequent studies showed that the analogues had the same or greater antiproliferative activity as 1,25(OH)2D3 (7-11). Because of the suggestion that 1,25(OH)2D3 might exert its antiproliferative actions in part by reducing ER concentrations (11), we examined whether ER+ and ER- cells were differentially affected by these analogues and whether combination with an antiestrogen would alter their efficacy.

We chose three cell lines for these studies, based on their level of ER expression: MCF-7 (high), BT-474 (low), and MDA-MB-453 (absent). Our data demonstrate the efficacy of selected 1,25(OH)2D3 analogues in inhibiting the proliferation of breast cancer cells *in vitro*. Treatment of cell cultures for 3 days resulted in inhibition of proliferation of all three cell lines in a dose-dependent manner; however, EB1089 was as much as 100-fold more effective than 1,25(OH)2D3 in BT-474 cells, and 16-ene,23-yne as much as 1000-fold more effective in MCF-7 cells. The ER- cell line, MDA-MB-453, was the most sensitive cell line to these analogues, with as much as 83% inhibition of proliferation occurring in response to 10^-6 M EB1089.

MCF-7 and BT-474 exhibited a biphasic response to 1,25(OH)2D3 and its analogues in the presence of E2, as low doses (10^-12 to 10^-10 M) were proliferative, whereas doses greater than 10^-9 M were antiproliferative. This biphasic behavior has also been noted in keratinocytes, where doses greater than 10^-8 M inhibited cell growth, whereas doses of 10^-9 M and less enhanced proliferation (16). This proproliferative effect of 1,25(OH)2D3 and its analogues in ER- and E2-responsive breast cancer cells could indicate that E2 blocks the antiproliferative action of 1,25(OH)2D3 on breast cancer cells, especially given that the stimulatory effect was not observed when MCF-7...
cells were treated with 1,25(OH)2D3 or EB1089 in the absence of E2.
If this is the case, this important interaction could have profound
implications for both the etiology of breast cancer and its treatment.
Current experiments in our laboratory are aimed at examining this
possibility and exploring mechanisms for this interaction.

We determined that each cell line expressed VDR mRNA and
protein. All four analogues bound to the VDR in all three cell lines
with high affinity comparable to 1,25(OH)2D3, however, contrary to
results in keratinocytes (17, 18), 16-ene bound with lower affinity
than 1,25(OH)2D3 in two of the three cell lines. In BT-474 cells,
16-ene,23-yne and EB1089 bound with higher affinity than
1,25(OH)2D3; in MCF-7 cells 16-ene, 16-ene,23-yne, and EB1089 did
so; and in MDA-MB-453 cells none of the analogues bound with
higher affinity than 1,25(OH)2D3. Interestingly, the ER- cell lines
had similar VDR mRNA and protein levels, whereas ER+ MDA-MB-
453 had lower mRNA, but generally higher protein levels, consistent
with their greater response to 1,25(OH)2D3 and the analogues. This
might indicate that E2 down regulates VDR expression in the ER+
cells, thus impeding the antiproliferative effects of 1,25(OH)2D3 and
the analogues.

The affinities of 1,25(OH)2D3 and the analogues correlated with the
antiproliferative actions in BT-474 and MCF-7 cells, whereas this was
not the case for MDA-MB-453 cells. Furthermore, the differences in
affinities for the VDR were relatively small (within one order of
magnitude) compared to the differences in antiproliferative potency
(several orders of magnitude). Such discrepancies between affinities
for the VDR and biological potency are observed in other systems and
could be secondary to a number of differences in the analogues. These
could include cellular uptake and metabolism, or induced confor-
mational changes in the VDR altering its binding to the retinoic acid
receptor RXR and/or DNA (19) or to cellular actions not mediated by
the VDR (20).

All three cell lines were inhibited by ICI 164,384, in a dose-
dependent manner, at concentrations greater than 10-9 M. ICI 164,384
is described as a pure antagonist of the ER (21). The fact that the
MDA-MB-453 cells responded consistently to this compound, albeit
not to the extent seen in ER+ cell lines, provides evidence that ICI
164,384 can mediate its actions through a non-ER mechanism in
addition to its classical actions through the ER. An alternate expla-
nation could be that MDA-MB-453 cells express a mutant but func-
tional ER that cannot bind the monoclonal antibody in the Abbott
Laboratories kit. Furthermore, the small but consistent stimulation of
proliferation by ICI 164,384 in BT-474 and MCF-7 suggests that ICI
164,384 might not be a pure antagonist in all cells.

Considerable data have accumulated to indicate that “cross-talk"
occurs among E2, ER, and antiestrogens and various growth factors,
growth factor receptors, nuclear factors, and signal transduction path-
ways in various tumor cell lines. It has been demonstrated that
pretreatment of transfected human adenocarcinoma cells with ICI
164,384 inhibited the effects of epidermal growth factor, transforming
growth factor-α, and E2 (22). Another study showed that antiestrogens
inhibited, whereas E2 stimulated, insulin-like growth factor-1-induced
AP-1-mediated transcription (23). Newton et al. (24) showed that ICI
182,780, another antiestrogen, can block both basal and insulin-like
growth factor-1-induced growth of pituitary tumor cells in the absence
of serum and E2, and they conclude from this and other experiments
that the ER is transcriptionally activated by intracellular peptide factor
pathways in the absence of E2 (24). In our current studies, the
antiproliferative actions of 1,25(OH)2D3, and EB1089 (data not
shown) were potentiated markedly by ICI 164,384, particularly in the
ER+ cells, MCF-7 and BT-474. This demonstration that ICI 164,384
converts the biphasic response in ER+ cells to one resembling that of
the ER- MDA-MB-453 cells suggests that E2 in an ER+ cell not only
blocks the antiproliferative action of 1,25(OH)2D3 and the analogues
but actually enhances its proliferative effects at low physiological
concentrations. These studies all indicate the potential role of E2 in

Fig. 5. Dose response to the antiestrogen ICI 164,384. Cells were treated with the
indicated concentrations of ICI 164,384 for 3 d, and then proliferation was measured using
[3H]thymidine incorporation. Data are expressed as a percentage of the control
values. Each point represents the mean of triplicates, and SDs were generally <8% (not greater
than 12%). Similar results were obtained in four separate experiments.

Fig. 6. Effect of 1,25(OH)2D3 in combination with 10-8 M ICI 164,384 on proliferation of breast cancer cells. Cells were treated with 1,25(OH)2D3 (□) at the indicated
concentrations, or 1,25(OH)2D3 + 10-8 M ICI 164,384 (●) for 3 d. Proliferation was measured using [3H]thymidine incorporation. Data are expressed as percentage of the control
values. Each point represents the mean of triplicates, and SDs were generally <8% (not greater than 17%). Similar results were obtained in two separate experiments.
mediating the response of ER$^+$ cells to growth factors and hormones such as 1,25(OH)$_2$D$_3$. Conceivably, physiological concentrations of E$_2$ and 1,25(OH)$_2$D$_3$ in vivo may synergize to promote tumor growth in women with ER$^+$ and VDR$^+$ breast cancers.

In conclusion, we have demonstrated that 1,25(OH)$_2$D$_3$ and the four analogues used in these studies bind the VDR of all three cell lines with high affinity and inhibit the proliferation of both ER$^+$ and ER$^-$ breast cancer cells in vitro, and that ICI 164,384 markedly potentiates this action. In addition, there is a differential response of breast cancer cells, based on ER expression. These findings suggest that dual therapy with a 1,25(OH)$_2$D$_3$ analogue and an antiestrogen may prove optimal for the chemoprevention or treatment of breast cancer, and indicate potential therapeutic possibilities for both ER$^+$ and ER$^-$ breast cancers.

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