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**Abstract:**
The purpose is to assess how estrogen (E2) alters the antiproliferative effects of 1,25(OH)2D3, and to determine how this interaction is modified in the presence of ICI164,384 (antiestrogen). IGF-I and II exhibit strong proliferative effects on breast cancer cells. The scope herein will determine whether 1,25(OH)2D3 affects IGF-I/R function, or production of its ligand IGF-II, and whether there is a difference in effects on ER+ and ER- cells. Anti-IGF-I/R Mab consistently elicited a decrease in proliferation of MCF-7 (ER+) cells E2, but not to MDA-MB-453 (ER-) cells. Anti-IGF-II Mab caused a decrease in proliferation in MCF-7 cells, but had no effect on MDA-MB-453 cells. 1,25(OH)2D3 at 10^-11 or 10^-8 M caused decreased proliferation only in MCF-7 cells in the presence of anti-IGF-II Mab; E2 overcame some of the decrease in proliferation by the Mab or 1,25(OH)2D3. The concentration of 1,25(OH)2D3 that stimulates MCF-7 cells (10^-11 M) unexplainedly stimulated IGF-I-induced IGF-I/R phosphorylation, and the antiproliferative concentration (10^-8 M) further stimulated phosphorylation, indicating that antiproliferative effects of 1,25(OH)2D3 might not be mediated through alteration of IGF-I/R autophosphorylation. More puzzling was the finding that ICI164,384 caused a further increase in stimulation of the autophosphorylation. MDA-MB-453 cells showed no stimulatory response of the IGF-I/R to IGF-I.

**Subject Terms:** Breast Cancer
IGF-I/R, IGF-II, Monoclonal Antibodies (Mab), Estrogen, Antiestrogens, Proliferation, Autophosphorylation

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Dr. Cheryl Love-Schimenti 11/20/97
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INTRODUCTION

Subject
1,25-dihydroxyvitamin D3 (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 vitamin D receptor (VDR) is widely expressed in a large variety of tissue types, including skin, endocrine, cardiac, immune, and hematopoietic cells, and that 1,25(OH)2D3 may be involved in the regulation of proliferation and differentiation of these cells [3]. Malignancies developing within many of these tissues contain VDR, and thus are likely to respond to 1,25(OH)2D3. 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].

Breast cancer patients with VDR+ tumors 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 [7-9]. The doses necessary to achieve tumor suppression, however, are likely to result in the development of hypercalcemia.

Nonhypercalcemic 1,25(OH)2D3 analogs have been developed in efforts 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 1,25(OH)2D3 and EB1089 [10]. 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 analogs in the treatment of breast cancer.

Estrogen (E2) is critically important for the growth of normal breast cells, as well as some breast cancer cells. Antiestrogens play an important role in the treatment of hormone-responsive breast cancers. Previous studies have demonstrated in vitro that tamoxifen increases the ability of certain 1,25(OH)2D3 analogs to inhibit breast cancer growth [9-10]. Recent studies have examined the effects of 1,25(OH)2D3 and some of the analogs on levels of ER expression, and one of these has demonstrated that 1,25(OH)2D3 and EB1089 downregulate ER expression in MCF-7 cells [10]. This effect was much stronger for EB1089 than 1,25(OH)2D3.

Purpose
I observed, in serum-free medium, that 1,25(OH)2D3 and its analogs elicit a biphasic response in ER+ (MCF-7 and BT-474) breast cancer cells, in that they stimulate proliferation at physiologic doses (10^-12 to 10^-10 M), but inhibit proliferation at higher doses (above 10^-9 M). However, 1,25(OH)2D3 and its analogs are antiproliferative in the ER- cell line, MDA-MB-453, at all doses above 10^-12 M. The antiestrogen ICI164,384, at a dose (10^-8 M) that had minimal antiproliferative effect when given singly to breast cancer cells, potentiated the antiproliferative effects of 1,25(OH)2D3 and its analogs in ER+ cells by 100- to 1000-fold, in essence converting the biphasic response of the ER+ cells to one resembling that of the ER- cells.

IGF-I and II exhibit strong proliferative effects on breast cancer cells [11-12]. Both are produced by breast stromal cells, while only IGF-II is also produced by breast epithelial cells [13]. Both growth factors can act through the IGF-I-R, which is found in most breast cancer cells [14], and is in fact overexpressed in many of these [15-16]. A tamoxifen compound has been found to induce a decrease in IGF-I binding sites in MCF-7 cells [17]. It is possible that E2 and 1,25(OH)2D3 interact to alter the proliferation of breast cancer cells
by controlling IGF-I-R activity or IGF production, and that antiestrogens may reverse these effects.

Initially, to address this possibility, I incubated MCF-7 cells with 1,25(OH)\textsubscript{2}D\textsubscript{3} and E\textsubscript{2}, in the presence or absence of ICI 164,384 for three days, then assayed IGF-I-stimulated IGF-I-R activation by measuring IGF-I-R autophosphorylation. 1,25(OH)\textsubscript{2}D\textsubscript{3} increased IGF-I-R tyrosine phosphorylation, and ICI164,384 blocked this effect, indicating that E\textsubscript{2} potentiates the pro-proliferative effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} by altering the activation of the IGF-I-R (see preliminary studies below).

These studies will indicate whether 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089 affect IGF-I-R expression or function, or the production of its ligand IGF-II, and whether there is a difference in these effects on the IGF-I-R and IGF-II in ER\textsuperscript{+} and ER\textsuperscript{-} breast cancer cells.

Scope of the Research
Numerous other studies have demonstrated interactions between E\textsubscript{2} and growth factors in breast cancer cells. In this project, the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and its nonhypercalcemic analog EB1089 on insulin-like growth factor receptor (IGF-I-R) expression and function and IGF-II production in breast cancer cells are being evaluated. The hypothesis being tested is that 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089 at physiologic doses enhance breast cancer cell proliferation by altering IGF-I-R functionality, e.g. autophosphorylation, through an E\textsubscript{2}-dependent mechanism. This possible enhancement of breast cancer cell proliferation by low doses of 1,25(OH)\textsubscript{2}D\textsubscript{3} and E\textsubscript{2}, and the marked potentiation of the antiproliferative actions of 1,25(OH)\textsubscript{2}D\textsubscript{3} when coupled with an antiestrogen, could have profound therapeutic implications. Data from these experiments will also indicate whether the presence of E\textsubscript{2} alters the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089 on the IGF-I-R expression or function, or IGF-II production, and whether ICI164,384 converts the effects on the ER\textsuperscript{+} cells to resemble these on the ER\textsuperscript{-} cells. The IGF-I-R and the ligand IGF-II were selected to investigate because IGF-I-R expression is increased in breast cancer cells, and IGF-I and IGF-II are strongly mitogenic for breast cancer cells. The major goal of the project is to assess how E\textsubscript{2} alters the antiproliferative effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089, and to determine how this interaction is modified in the presence of the antiestrogen ICI 164,384. I expect to observe differences in ER\textsuperscript{+} and ER\textsuperscript{-} cells in IGF-I-R concentration or phosphorylation, or in the level of production of IGF-II, because of the differences observed in the responses of ER\textsuperscript{+} and ER\textsuperscript{-} cells to different concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089. I anticipate relating these differences to the various hormonal combinations to be examined. One of the major questions asked in this project is why 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089 are proliferative at low concentrations but antiproliferative at higher concentrations, and how E\textsubscript{2} potentiates the proliferative but blocks the antiproliferative effects of these compounds. I can then more clearly interpret the role of E\textsubscript{2} and the estrogen receptor in mediating the differences between the cell lines, regarding the changes observed in the IGF-I-R or IGF-II.

Background of Previous Studies
Before beginning the studies outlined in the current grant, a number of preliminary experiments had been completed, and several others were ongoing.

1. Development of serum-free culture conditions for breast cancer cells.
   Different lots of serum contain variable amounts of estrogen, vitamin D metabolites, vitamin D binding proteins, IGFs, IGF binding proteins, and many other growth factors. For this reason, I had to first develop a serum-free system in which to perform our assays in a controlled manner. I adapted our cell lines to grow in medium supplemented with ITS\textsuperscript{+} (insulin, transferrin, selenium) for the period of each assay, and all in vitro proliferation assays were accomplished with this supplement.
2. Effect of 1,25(OH)_2D_3 and 1,25(OH)_2D_3 analogs on proliferation of MCF-7, BT-474, and MDA-MB-453 cells.

Three cell lines were chosen for varying ER expression - MCF-7 (ER\textsuperscript{high}), BT-474 (ER\textsuperscript{low}), and MDA-MB-453 (ER\textsuperscript{+}) - and analyzed for their proliferative responses to 1,25(OH)_2D_3 and four of its analogs. The two ER\textsuperscript{+} and E\textsubscript{2}-responsive cell lines, MCF-7 and BT-474, were supplemented with 10\textsuperscript{-10} M E\textsubscript{2} in all assays. MDA-MB-453, which is ER\textsuperscript{+} and E\textsubscript{2}-unresponsive, did not receive E\textsubscript{2}. 1,25(OH)_2D_3 and the 4 analogs tended to stimulate the proliferation of the ER\textsuperscript{+} cells at doses below 10\textsuperscript{-9} M, while inhibiting proliferation at doses above 10\textsuperscript{-9} M. This proproliferative effect is modest, between 25-50%, but is consistently seen at doses below 10\textsuperscript{-9} M. In contrast, only 10\textsuperscript{-12} M 1,25(OH)_2D_3 and the analogs occasionally stimulated proliferation of MDA-MB-453 by less than 25%, while doses above that were antiproliferative. DNA measurements were made for all experiments, and the results paralleled those obtained with [\textsuperscript{3}H]-thymidine incorporation. Based on these data, it appeared that 1,25(OH)_2D_3 and its analogs at physiologic doses might actually enhance breast cancer cell proliferation through an E\textsubscript{2}-dependent mechanism.

3. Effect of ICI164,384 alone and in combination with 1,25(OH)_2D_3 and its analogs on growth of MCF-7, BT-474, and MDA-MB-453 cells.

To determine whether the antiestrogen ICI164,384 could synergize with 1,25(OH)_2D_3 to inhibit breast cancer cell proliferation, a series of proliferation assays were performed using this compound. The antiestrogen ICI164,384 had an antiproliferative effect on all three cell lines (including the ER\textsuperscript{-} line, MDA-MB-453). Interestingly, in combination with 1,25(OH)_2D_3, ICI164,384 enhanced the antiproliferative action of 1,25(OH)_2D_3 on the ER\textsuperscript{+} cells MCF-7 and BT-474, converting their response to one resembling that of the ER\textsuperscript{-} cell line MDA-MB-453. However, even in the ER\textsuperscript{-} line, MDA-MB-453, ICI164,384 potentiated the antiproliferative actions of 1,25(OH)_2D_3, suggesting that ICI164,384 might have effects other than through the ER. These results indicated the potential for utilizing the 1,25(OH)_2D_3 analogs as antiproliferative agents in the treatment of breast cancer in a setting in which the proproliferative actions of low doses are blocked or not apparent.

4. Effect of 1,25(OH)_2D_3 in the presence and absence of ICI164,384 on tyrosine phosphorylation of IGF-I-R in MCF-7 cells.

To address the possibility that 1,25(OH)_2D_3 in combination with E\textsubscript{2} could be altering growth factor activity or growth factor receptor activation, preliminary experiments were performed to examine the effect of 1,25(OH)_2D_3 plus E\textsubscript{2}, in the presence and absence of ICI 164,384, on the tyrosine phosphorylation of IGF-I-R. MCF-7 cells were incubated with 1,25(OH)_2D_3 and E\textsubscript{2}, in the presence and absence of ICI164,384, for 3 days, in medium with insulin excluded. This exclusion was necessary in order to see effects on IGF-I-stimulated IGF-I-R phosphorylation. We then assayed the ability of these compounds to alter IGF-I-stimulated IGF-I-R tyrosine phosphorylation. 1,25(OH)_2D_3 appeared to increase tyrosine phosphorylation, and the antiestrogen ICI164,384 increased this autophosphorylation, opposing my hypothesis that 1,25(OH)_2D_3 enhances breast cancer cell proliferation through an E\textsubscript{2}-dependent mechanism. This is a troublesome result that is unexplainable to date.

5. Displacement studies for presence of VDR (Scatchard analysis), and mRNA analysis.

To ensure that all 3 cell lines expressed the VDR, and therefore, the observed effects were mediated through that receptor, radioactive displacement studies were performed with increasing amounts of non-radioactive 1,25(OH)_2D_3, and all three cell lines expressed the VDR at relatively high levels. Interestingly, the ER\textsuperscript{-} cell line MDA-MB-453 expressed almost twice as much of the VDR as the ER\textsuperscript{+} cell lines. When mRNA levels of the VDR were measured, all three cell lines had virtually the same levels, with the possibility that MDA-MB-453 had a slightly lower level than the other two cell lines.

Studies from our own laboratory as well as others indicated that EB1089 had a greater antiproliferative effect with less toxicity in vivo than 1,25(OH)_{2}D_{3}. We wished to determine the highest dose of EB1089 that could be given to nude mice without evidence of hypercalcemia or toxicity. To determine the effects of 0-2 mg/kg body weight of EB1089 on serum calcium and body weight, groups of 3 mice were treated daily for 7 days, and serum calcium was measured on days 1, 3, and 7. One-half of the mice were treated by oral gavage, and the other half by intraperitoneal (i.p.) injection of EB1089 or vehicle (propylene glycol). All mice were maintained for the duration of the experiment on a low calcium diet so that any hypercalcemia that developed could be related to the treatment by EB1089. None of the mice developed hypercalcemia, even at the 2 mg/kg body weight dose. All mice in the i.p. treatment groups, however, lost substantial body weight (as much as 25%) in 7 days. In addition, on autopsy, the abdominal cavities appeared damaged by the treatment. The mice treated by oral gavage, on the other hand, did not lose weight, or show any other signs of harmful effects from EB1089 treatment. Based on this study, I am prepared to begin studies on the effects of EB1089 on established breast cancer tumors in mice during the third year of the grant period, after the in vitro studies have been completed.

BODY

Experimental Methods

A. Cell culture. Cells were plated in 5% FCS-supplemented DMEM medium, switched after two days to serum-free, phenol red-free DMEM, supplemented with insulin, transferrin, selenium (ITS+), and twenty-four hours later, they were treated with 10^{-10} M E_{2}, 10^{-11} M or 10^{-8} M 1,25(OH)_{2}D_{3} or EB1089, ICI164,384, or vehicle (100% ethanol), and combinations of compounds. Medium was changed and fresh compounds added after 48 hours. Growth was assessed by [³H]-thymidine incorporation, and activation of the IGF-I-R was assessed by and ELISA method. These methods were previously well established in our laboratory.

B. Measurement of IGF-I-R by Western analysis. Separation and blotting of samples is currently being performed as for standard SDS-PAGE analysis, using comparable methods described for Western ligand-binding, but with 8% acrylamide gel and SDS-PAGE buffers containing reducing agents. Primary antibody has been obtained from Oncogene Science (Cambridge, MA), and is being used at their recommended dilutions. Detection will be performed using enhanced chemiluminescence (ECL) according to manufacturer’s directions (Amersham Life Science, Arlington Hts., IL), and quantified by densitometry.

C. Measurement of effects of anti-IGF-I-R and IGF-II monoclonal antibodies on proliferation of breast cancer cells. Anti-IGF-II antibodies were obtained from Upstate Biotechnology (Lake Placid, New York), and anti-IGF-I-R from Oncogene Science (Cambridge, MA) and dose-response studies were first performed with each of the antibodies. Cells were then treated with 10^{-10} M E_{2}, 10^{-11} M or 10^{-8} M 1,25(OH)_{2}D_{3}, ICI164,384, or vehicle (100% ethanol), and combinations of compounds, +/- the highest dose of the monoclonal antibodies.

D. Measurement of mRNA levels for IGF-I-R and IGF-II. Total RNA was prepared at the time of cell harvest, as previously described [20], after treatment of cells as above under cell culture. Cells were then disrupted in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol), and extracted with phenol-chloroform. RNA was precipitated with ethanol, and analysis is being performed as follows.

1. Northern analysis. This method is being used first to measure IGF-I-R and IGF-II RNA. RNA samples (20-30 mg) were run on 1% agarose-formaldehyde gels, and
transblotted onto nylon membranes. Blots were probed with human cDNAs for IGF-I-R and IGF-II (gift from Dr. Charles T. Roberts, Jr.), and hybridization detected by autoradiography and quantitated by densitometry. Results will be normalized to the amount of 18S RNA on the blot, detected with a probe to the 18S RNA, or to the human glyceraldehyde-3-phosphate dehydrogenase (GAPD) transcript using a commercially available 1.1 kbp probe (Clontech).

2. Ribonuclease protection assay. Currently, appropriately sized fragments of these probes are being subcloned into a pGEM vector to permit the preparation of riboprobes for RNase protection assays. Transcription of the riboprobes will be performed using a kit, according to the manufacturer’s instructions (Promega, Madison, WI), and the probes gel purified prior to use. Thirty mg RNA samples will be incubated with 2-4 x 10^5 cpm of the riboprobes overnight at 45°C prior to RNase digestion and separation of the products on a sequencing gel. 5 x 10^4 cpm of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) riboprobe will be included in the incubation, and used to normalize the amount of mRNA in each sample. The protected bands will be detected by autoradiography and quantitated by densitometry. RNase protection analysis provides approximately one order of magnitude greater sensitivity than Northern analysis, and is well established in our laboratory.

E. In vitro kinase assay for IGF-I-R phosphorylation. Cells were treated as described above under cell culture for 3 days. Medium was changed and allowed to equilibrate for 15 min. at 37°C in a CO2 incubator. IGF-I at a concentration ranging of 10 nM was added for 20 min. to the cells (time was determined in dose-reponse and timed studies). The reaction was then stopped by placing cells on ice, rapidly aspirating the medium, and washing with 2 ml ice cold PBS. This wash was repeated, then 0.5 ml of ice cold solubilization solution (50 mM HEPES, pH 7.6, 1% Triton X-100, 0.1 mM AEBSF, 2 mM sodium orthovanadate) was added. Cells were scraped from the plate, transferred to a microfuge tube, and incubated for 1 hr. at 4°C. Cells were microfuged for 15 min., and the lysate transferred to new tubes. Protein concentration was determined by the Bradford protein assay. Samples were frozen at -80°C until used.

96-well Nunc ImmunoPlate I Maxisorb plates were coated with 100 ml (5.0 mg/ml) of a-IR3 (anti-IGF-I-R MAb, Oncogene Science, Cambridge, MA) in 50 mM sodium bicarbonate, pH 9.0 at 4°C for 18-24 hours. Plates were washed 3x with TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20) and blocked with 150 ml of TBST containing 1% BSA (Buffer A) for 30 min. at 56°C. Wells were washed 3x with TBST, then 100 ml cell lysate (25-30 µg) was added, and wells were incubated for 2 hr. at 22°C. Wells were washed 5x with TBST, then 100 ml of 0.3 mg/ml biotinylated anti-phosphotyrosine MAb (Upstate Biotechnology Incorporated, Lake Placid, New York) was added in Buffer B (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.05% Tween-20, 1% BSA, 2 mM vanadate, 1 mg/ml bimatracin, 0.1 mM AEBSF) for 2 hr. at 22°C. After 5 washes with TBST, 100 ml of 0.1 mg/ml peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) in Buffer B was added for 30 min. at 22°C. Wells were again washed 5x with TBST, then incubated for 30 min. with 100 ml TMB (Kirkegaard & Perry, Gaithersburg, MD) mixed 15 min. before adding to plates. Intensification of blue color signals the reaction. Reaction was stopped after approximately 5 min. with 100 ml 1 N NH₄PO₄, and wells were read at OD₄₅₀ nm.

Assumptions and Hypothesis
Concentrations of 1,25(OH)₂D₃ which stimulated proliferation (10⁻¹¹ M) in the presence of E₂, and higher concentrations which were antiproliferative on both ER⁺ and ER⁻ cells (10⁻⁸ M) were used in all studies. Differences were expected in ER⁺ and ER⁻ cells in IGF-I-R phosphorylation, and in the level of production of IGF-II, because of the differences previously observed in the responses of ER⁺ and ER⁻ cells to different concentrations of 1,25(OH)₂D₃ and EB1089 in the proliferation assays. I anticipated relating these differences to the various hormonal combinations examined. The hypothesis is that
1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089 at physiologic doses enhance breast cancer cell proliferation by altering IGF-I-R functionality, e.g. autophosphorylation, through an E\textsubscript{2}-dependent mechanism in ER\textsuperscript{+} cells; E\textsubscript{2} alters these antiproliferative effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB1089; and the interaction is modified in the presence of the antiestrogen ICI164,384.

Procedures
The procedures for the first year's experiments involved (1) exploring the regulation of IGF-I-R function, and IGF-II production in MCF-7 (ER\textsuperscript{+}) and MDA-MB-453 (ER\textsuperscript{−}) breast cancer cells by 1,25(OH)\textsubscript{2}D\textsubscript{3}; (2) determining the ability of 1,25(OH)\textsubscript{2}D\textsubscript{3} to alter these factors; and (3) determining the role of E\textsubscript{2} in modulating the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on IGF-I-R function and IGF-II production. Data from these experiments were expected to establish whether the presence of E\textsubscript{2} alters the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on the IGF-I-R function, or IGF-II production in ER\textsuperscript{+} cells, and whether ICI164,384 would convert the effects on the ER\textsuperscript{+} cells to resemble those of the ER\textsuperscript{−} MDA-MB-453 cells.

Results and Discussion
The first year's work was designed to evaluate the functional capacity of the IGF-I-R and the production of IGF-II in the two cell lines, MCF-7 and MDA-MB-453, and to then determine the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on these factors. Since ICI164,384 had been shown in previous studies to convert the proliferative response of ER\textsuperscript{+} cells to one resembling that of the ER\textsuperscript{−} cells, 1,25(OH)\textsubscript{2}D\textsubscript{3} was expected, in the presence of E\textsubscript{2}, to stimulate IGF-II production and IGF-I-R activation in MCF-7 cells, while having little or no such effect on MDA-MB-453 cells. The proliferative response that we have observed in the MCF-7 cells to low doses of 1,25(OH)\textsubscript{2}D\textsubscript{3} and the analogs is in the range of 25-50%, and is consistently seen at doses below 10^{-9} M, although the extent varies somewhat from experiment to experiment.

Anti-IGF-I-R monoclonal antibodies (Mab) were added to cultures treated with or without 10^{-10} M E\textsubscript{2} to determine their effect on the MCF-7 (ER\textsuperscript{+}) cells, which consistently responded with a decrease in proliferation (figure 1), and on MDA-MB-453 (ER\textsuperscript{−}) cells, which showed no response to the antibody (figure 1). This indicated the possibility that the hypothesis regarding the role of E\textsubscript{2} in interfering with the antiproliferative activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} was correct. Anti-IGF-II Mab were next used to determine whether the cells were producing IGF-II, and whether this growth factor could be playing a role in the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} on the cells. As demonstrated in figure 2, a clean dose-response was obtained in MCF-7 cells when treated with increasing amounts of anti-IGF-II Mab. A dose-response study was performed on both cell lines to determine the optimal dose to use for further studies. When MCF-7 cells were treated with the highest dose of anti-IGF studies, there was a significant decrease in proliferation, and the presence of estrogen appeared to have little effect on this decrease (figure 3). Finally, the effects of anti-IGF-II Mab in the presence or absence of E\textsubscript{2}, 10^{-11} or 10^{-8} M 1,25(OH)\textsubscript{2}D\textsubscript{3} on the proliferation of MCF-7 and MDA-MB-453 cells were examined. As in previous studies, 1,25(OH)\textsubscript{2}D\textsubscript{3} caused a decrease in proliferation of both cell lines, while in these new studies anti-IGF-II caused a decrease in proliferation only in MCF-7 cells, and E\textsubscript{2} appeared to overcome some of this decrease caused by the antibody and by 1,25(OH)\textsubscript{2}D\textsubscript{3} (figures 4a and 4b).

One problem encountered with both the anti-IGF-II antibody and the IGF-I-R antibody was expense. Using a relatively high dose of each antibody resulted in only about a 33-50% decrease in proliferation of MCF-7 with anti-IGF-I-R, and about a 10-15% decrease with anti-IGF-II. The decreases, however, were consistent in repeat studies. Unfortunately, these antibodies are extremely expensive, and therefore, some studies have not yet been performed and some have been shortened as to numbers of samples.

The proproliferative dose chosen for these studies (10^{-11} M) almost always had a consistently proproliferative effect. The concentration of 1,25(OH)\textsubscript{2}D\textsubscript{3} that was...
stimulatory to MCF-7 cells (10^{-11} M) stimulated IGF-I-induced IGF-I-R phosphorylation. The most confusing result was that the concentration of 1,25(OH)_2D_3 which is antiproliferative (10^{-8} M), however, further stimulated phosphorylation, indicating that the antiproliferative effects of 1,25(OH)_2D_3 in breast cancer cells might not be mediated through alteration of IGF-I-R phosphorylation (figures 5a and 5b). Even more puzzling was the finding that ICI164,384 caused a further increase in stimulation of the autophosphorylation of the IGF-I-R. MDA-MB-453 cells showed no stimulatory response of the IGF-I-R to IGF-I.

Recommendations in Relation to Statement of Work
Failure to see differences between the ER^+ and ER^- cells (i.e. 1,25(OH)_2D_3 stimulated or decreased IGF-I-R and/or IGF-II production in both MCF-7 and MDA-MB-453 cell types) would have led us to explore alternative mechanisms, such as IGF binding protein production or post-receptor events in the MAP kinase or insulin receptor substrate-1 (IRS-1) pathway, that could modulate the role of the IGF/IGF-R system in mediating the proproliferative actions of 1,25(OH)_2D_3 in the ER^+ cells. Although there were differences between the cell lines, the results by MCF-7 cells to IGF-I-stimulated IGF-I-R autophosphorylation, in opposition to all other experiments, make it important to explore some of these other post-receptor events.

In addition, insulin is known to modulate the response of cells, (e.g. keratinocytes) to 1,25(OH)_2D_3. Since insulin must be removed from these experiments in order to see the effects of IGF-I on the IGF-I-R, the early dose/response proliferation experiments may need to be repeated to 1,25(OH)_2D_3 and the analogs in the absence of insulin. This will allow the determination of whether insulin alters the response of breast cancer cells to 1,25(OH)_2D_3 and E_2.

CONCLUSIONS
1,25(OH)_2D_3 effectively blocks the proliferation in both cell lines examined, however, E_2 only blocks proliferation in the ER^+ MCF-7 cells. Monoclonal antibodies to IGF-I-R and IGF-II decreased proliferation only in the MCF-7 cells. The anti-IGF-I-R Mab data, in MCF-7 cells +/-E_2 suggests that only MCF-7 cells are partially dependent on the activation of the IGF-I-R for proliferation. The anti-IGF-II Mab also decreased the antiproliferative effects of 1,25(OH)_2D_3, but not to the extent of the IGF-I-R Mab, and E_2 blocked the ability of IGF-I to stimulate the autophosphorylation of the IGF-I-R.

Although the IGF-I-R may be important in IGF-I- or IGF-II-stimulated proliferation in MCF-7 (ER^+ cells), our data indicate that the mechanism by which E_2 increases and 1,25(OH)_2D_3 decreases the proliferation of MCF-7 cells may not be through changes in the IGF-I-R, or at least not entirely through changes in this receptor, and the mechanism in fact by which E_2 and 1,25(OH)_2D_3 have opposite effects is under active investigation.

REFERENCES


Figure 1

Effect of Anti-IGF-1-R Antibody on Proliferation of Breast Cancer Cells

![Graph showing the effect of Anti-IGF-1-R antibody on the proliferation of MCF-7 and MDA-MB-453 cells. The x-axis represents the presence or absence of estrogen and anti-IGF-1-R. The y-axis represents the counts per minute (CPM).]
Figure 2

Effect of Anti-IGF-II MAb on Proliferation: Dose-Response

- Control
- + 10 ng/ml IGF-II MAb
- + 20 ng/ml IGF-II MAb
- + 40 ng/ml IGF-II MAb

MCF-7

MDA-453
Figure 3
Effects of Anti-IGF-II Antibody in the Presence/Absence of Estrogen

Estrogen
-  +  -  +
Anti-IGF-II  -  -  +  +

CPM

- MCF-7
- MDA-MB-453
Figure 4a
Effects of Anti-IGF-II MAb on MCF-7 in Presence of E2, and $10^{-11}$ or $10^{-8}$ M $1,25(OH)_2D_3$

![Bar graph showing CPM levels across different treatments A, B, and C.](image)

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<th>A</th>
<th>B</th>
<th>C</th>
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<td>- - + +</td>
<td>- - + +</td>
<td>- - + +</td>
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<tr>
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<td>- + - +</td>
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<td>+ + + +</td>
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A=Vehicle  
B=$10^{-11}$ M 1,25  
C=$10^{-8}$ M 1,25
Effects of Anti-IGF-II MAb on MDA-MB-453 in the Presence of E2, and $10^{-11}$ or $10^{-8}$ M $1,25(\text{OH})_2\text{D}_3$

- A = Vehicle
- B = $10^{-11}$ M $1,25\text{D}_3$
- C = $10^{-8}$ M $1,25\text{D}_3$

E2
- - ++
- + - +
- - - -

Anti-IGF-II MAb
- ++
- + +
- +

$1,25(\text{OH})_2\text{D}_3$
- -
- +
- + ++
Figure 5a

Effect of Estrogen and 1,25(OH)2D3 on IGF-I-Stimulated IGF-I-R Tyrosine Phosphorylation in MCF-7 Cells
Figure 5b

Effect of Estrogen and 1,25(OH)2D3 on IGF-I-Stimulated IGF-I-R Tyrosine Phosphorylation in MDA-MB-453 Cells

OD450

control 1.25:11 M 1.25:8 M ICI 182780 ICI +11 M ICI +8 M

no E2 + E2