

AD _____

GRANT NUMBER DAMD17-96-1-6218

TITLE: Estrogen-Modulated Response of Breast Cancer to Vitamin D
and its Analogs: Role of IGF

PRINCIPAL INVESTIGATOR: Cheryl D. Love-Schimenti, Ph.D.

CONTRACTING ORGANIZATION: Northern California Institute
for Research and Education
San Francisco, CA 94121

REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 2

19980310 074

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1997	3. REPORT TYPE AND DATES COVERED Annual (1 Oct 96 - 30 Sep 97)	
4. TITLE AND SUBTITLE Estrogen-Modulated Response of Breast Cancer to Vitamin D and its Analogs: Role of IGF			5. FUNDING NUMBERS DAMD17-96-1-6218	
6. AUTHOR(S) Cheryl D. Love-Schimenti, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northern California Institute for Research and Education San Francisco, CA 94121			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) <p>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.</p>				
14. SUBJECT TERMS Breast Cancer IGF-I-R, IGF-II, Monoclonal Antibodies (Mab), Estrogen, Antiestrogens, Proliferation, Autophosphorylation			15. NUMBER OF PAGES 20	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

cls In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Dr. Cheryl Love-Schimenti 11/20/97
PI - Signature Date

TABLE OF CONTENTS

	Page Number
1. FRONT COVER	1
2. REPORT DOCUMENTATION FORM	2
3. FOREWORD	3
4. TABLE OF CONTENTS	4
5. INTRODUCTION	5-8
6. BODY	8-11
7. CONCLUSIONS	11
8. REFERENCES	11-13
9. APPENDICES	14-20

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 $1,25(\text{OH})_2\text{D}_3$ and E_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. $1,25(\text{OH})_2\text{D}_3$ increased IGF-I-R tyrosine phosphorylation, and ICI164,384 blocked this effect, indicating that E_2 potentiates the pro-proliferative effects of $1,25(\text{OH})_2\text{D}_3$ by altering the activation of the IGF-I-R (see preliminary studies below).

These studies will indicate whether $1,25(\text{OH})_2\text{D}_3$ 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^+ and ER^- breast cancer cells.

Scope of the Research

Numerous other studies have demonstrated interactions between E_2 and growth factors in breast cancer cells. In this project, the effects of $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ and EB1089 at physiologic doses enhance breast cancer cell proliferation by altering IGF-I-R functionality, e.g. autophosphorylation, through an E_2 -dependent mechanism. This possible enhancement of breast cancer cell proliferation by low doses of $1,25(\text{OH})_2\text{D}_3$ and E_2 , and the marked potentiation of the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ when coupled with an antiestrogen, could have profound therapeutic implications. Data from these experiments will also indicate whether the presence of E_2 alters the effects of $1,25(\text{OH})_2\text{D}_3$ and EB1089 on the IGF-I-R expression or function, or IGF-II production, and whether ICI164,384 converts the effects on the ER^+ cells to resemble these on the ER^- 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_2 alters the antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$ 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^+ and ER^- 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^+ and ER^- cells to different concentrations of $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ and EB1089 are proliferative at low concentrations but antiproliferative at higher concentrations, and how E_2 potentiates the proliferative but blocks the antiproliferative effects of these compounds. I can then more clearly interpret the role of E_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⁺ (insulin, transferrin, selenium) for the period of each assay, and all *in vitro* proliferation assays were accomplished with this supplement.

2. Effect of $1,25(\text{OH})_2\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ 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^{high}), BT-474 (ER^{low}), and MDA-MB-453 (ER^-) - and analyzed for their proliferative responses to $1,25(\text{OH})_2\text{D}_3$ and four of its analogs. The two ER^+ and E_2 -responsive cell lines, MCF-7 and BT-474, were supplemented with 10^{-10} M E_2 in all assays. MDA-MB-453, which is ER^- and E_2 -unresponsive, did not receive E_2 . $1,25(\text{OH})_2\text{D}_3$ and the 4 analogs tended to stimulate the proliferation of the ER^+ cells at doses below 10^{-9} M, while inhibiting proliferation at doses above 10^{-9} M. This proproliferative effect is modest, between 25-50%, but is consistently seen at doses below 10^{-9} M. In contrast, only 10^{-12} M $1,25(\text{OH})_2\text{D}_3$ 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 [^3H]-thymidine incorporation. Based on these data, it appeared that $1,25(\text{OH})_2\text{D}_3$ and its analogs at physiologic doses might actually enhance breast cancer cell proliferation through an E_2 -dependent mechanism.

3. Effect of ICI164,384 alone and in combination with $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ 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^- line, MDA-MB-453). Interestingly, in combination with $1,25(\text{OH})_2\text{D}_3$, ICI164,384 enhanced the antiproliferative action of $1,25(\text{OH})_2\text{D}_3$ on the ER^+ cells MCF-7 and BT-474, converting their response to one resembling that of the ER^- cell line MDA-MB-453. However, even in the ER^- line, MDA-MB-453, ICI164,384 potentiated the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$, suggesting that ICI164,384 might have effects other than through the ER. These results indicated the potential for utilizing the $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$ in the presence and absence of ICI164,384 on tyrosine phosphorylation of IGF-I-R in MCF-7 cells.

To address the possibility that $1,25(\text{OH})_2\text{D}_3$ in combination with E_2 could be altering growth factor activity or growth factor receptor activation, preliminary experiments were performed to examine the effect of $1,25(\text{OH})_2\text{D}_3$ plus E_2 , in the presence and absence of ICI 164,384, on the tyrosine phosphorylation of IGF-I-R. MCF-7 cells were incubated with $1,25(\text{OH})_2\text{D}_3$ and E_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. $1,25(\text{OH})_2\text{D}_3$ appeared to increase tyrosine phosphorylation, and the antiestrogen ICI164,384 increased this autophosphorylation, opposing my hypothesis that $1,25(\text{OH})_2\text{D}_3$ enhances breast cancer cell proliferation through an E_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 $1,25(\text{OH})_2\text{D}_3$, and all three cell lines expressed the VDR at relatively high levels. Interestingly, the ER^- cell line MDA-MB-453 expressed almost twice as much of the VDR as the ER^+ 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.

6. In vivo treatment of nude mice with EB1089: preliminary and toxicity studies.

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)₂D₃. 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 E₂, 10⁻¹¹ M or 10⁻⁸ M 1,25(OH)₂D₃ 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 E₂, 10⁻¹¹ M or 10⁻⁸ M 1,25(OH)₂D₃, 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 $2-4 \times 10^5$ cpm of the riboprobes overnight at 45°C prior to RNase digestion and separation of the products on a sequencing gel. 5×10^4 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 CO_2 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 μl (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 μl of TBST containing 1% BSA (Buffer A) for 30 min. at 56°C . Wells were washed 3x with TBST, then 100 μl 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 μl 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 bacitracin, 0.1 mM AEBSF) for 2 hr. at 22°C . After 5 washes with TBST, 100 μl 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 μl 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 μl 1 N NH_3PO_4 , and wells were read at $\text{OD}_{450 \text{ nm}}$.

Assumptions and Hypothesis

Concentrations of $1,25(\text{OH})_2\text{D}_3$ which stimulated proliferation (10^{-11} M) in the presence of E_2 , 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 $1,25(\text{OH})_2\text{D}_3$ and EB1089 in the proliferation assays. I anticipated relating these differences to the various hormonal combinations examined. The hypothesis is that

1,25(OH)₂D₃ and EB1089 at physiologic doses enhance breast cancer cell proliferation by altering IGF-I-R functionality, e.g. autophosphorylation, through an E₂-dependent mechanism in ER⁺ cells; E₂ alters these antiproliferative effects of 1,25(OH)₂D₃ 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⁺) and MDA-MB-453 (ER⁻) breast cancer cells by 1,25(OH)₂D₃; (2) determining the ability of 1,25(OH)₂D₃ to alter these factors; and (3) determining the role of E₂ in modulating the effects of 1,25(OH)₂D₃ on IGF-I-R function and IGF-II production. Data from these experiments were expected to establish whether the presence of E₂ alters the effects of 1,25(OH)₂D₃ on the IGF-I-R function, or IGF-II production in ER⁺ cells, and whether ICI164,384 would convert the effects on the ER⁺ cells to resemble those of the ER⁻ 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)₂D₃ on these factors. Since ICI164,384 had been shown in previous studies to convert the proliferative response of ER⁺ cells to one resembling that of the ER⁻ cells, 1,25(OH)₂D₃ was expected, in the presence of E₂, 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 proproliferative response that we have observed in the MCF-7 cells to low doses of 1,25(OH)₂D₃ 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 E₂ to determine their effect on the MCF-7 (ER⁺) cells, which consistently responded with a decrease in proliferation (figure 1), and on MDA-MB-453 (ER⁻) cells, which showed no response to the antibody (figure 1). This indicated the possibility that the hypothesis regarding the role of E₂ in interfering with the antiproliferative activity of 1,25(OH)₂D₃ 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)₂D₃ 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 E₂, 10⁻¹¹ or 10⁻⁸ M 1,25(OH)₂D₃ on the proliferation of MCF-7 and MDA-MB-453 cells were examined. As in previous studies, 1,25(OH)₂D₃ 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 E₂ appeared to overcome some of this decrease caused by the antibody and by 1,25(OH)₂D₃ (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)₂D₃ 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 $1,25(\text{OH})_2\text{D}_3$ which is antiproliferative (10^{-8} M), however, further stimulated phosphorylation, indicating that the antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$ 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 IC164,384 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. $1,25(\text{OH})_2\text{D}_3$ 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 proliferative actions of $1,25(\text{OH})_2\text{D}_3$ 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 $1,25(\text{OH})_2\text{D}_3$. 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 $1,25(\text{OH})_2\text{D}_3$ and the analogs in the absence of insulin. This will allow the determination of whether insulin alters the response of breast cancer cells to $1,25(\text{OH})_2\text{D}_3$ and E₂.

CONCLUSIONS

$1,25(\text{OH})_2\text{D}_3$ effectively blocks the proliferation in both cell lines examined, however, E₂ 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 +/- E₂ 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 $1,25(\text{OH})_2\text{D}_3$, but not to the extent of the IGF-I-R Mab, and E₂ 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 E₂ increases and $1,25(\text{OH})_2\text{D}_3$ 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 E₂ and $1,25(\text{OH})_2\text{D}_3$ have opposite effects is under active investigation.

REFERENCES

1. Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. An overview of 61 randomized trials among 28,896 women. 1988. *New Engl. J. Med.* 319:1681-92.
2. Bikle DD. 1992. Vitamin D: new actions, new analogs, new therapeutic potential. *Endocrine Reviews.* 13:765-84.

3. Walters MR. 1992. Newly identified actions of the vitamin D endocrine system. *Endocrine Reviews*. 13:719-64.
4. Eisman JA, Martin TJ, MacIntyre I, Moseley JM. 1979. 1,25-dihydroxyvitamin-D-receptor in breast cancer cells. *Lancet*. 2:1335-6.
5. Berger U, Wilson P, McClelland RA, Colston K, Haussler MR, Pike JW, and Coombes RC. 1991. Immunocytochemical detection of 1,25-dihydroxyvitamin D₃ receptor in breast cancer. *Cancer Res*. 47:6793-99.
6. Colston KW, Berger U, Coombes RC. 1989. Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet*. 1:188-91.
7. Abe J, Nakano AT, Nishii Y, Matsumoto T, Ogata E, Ikeda K. 1991. A novel D₃ analog, 22-oxa-1,25-dihydroxyvitamin D₃, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology*. 129:832-7.
8. Eisman JA, Barkla DH, Tutton PJM. 1987. Suppression of *in vivo* growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D₃. *Cancer Res*. 47:21-5.
9. Vink-van Wijngaarden T, Pols HA, Buurman CJ, van den Bemd GJ, Dorssers LC, Birkenhager JC, van Leeuwen JP. 1994. Inhibition of breast cancer cell growth by combined treatment with vitamin D₃ analogues and tamoxifen. *Cancer Res*. 54:5711-17.
10. James SY, Mackay AG, Binderup L, Colston KW. 1994. Effects of a new synthetic vitamin D analogue, EB1089, on the oestrogen-responsive growth of human breast cancer cells. *J. Endocrinol*. 141:555-63.
11. Osborne CK, Coronado EB, Kitten LJ, Arteaga CL, Fuqua SAW, Ramasharma K, Mashall M, Li CH. 1989. Insulin-like growth factor II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Mol. Endocrinol*. 3:1701-09.
12. Arteaga CL, Osborne CK. 1989. Growth inhibition of human breast cancer cells *in vitro* with an antibody against the Type I somatomedin receptor. *Cancer Res*. 49:6237-41.
13. Paik S. 1992. Expression of IGF-I and IGF-II mRNA in breast tissue. *Breast Cancer Res. Treat.* 22:31-8.
14. Cullen KJ, Yee D, Sly WS, Perdue J, Hampton B, Lippman ME, Rosen N. 1990. Insulin-like growth factor receptor expression and function in human breast cancer. *Cancer Res*. 50:48-53.
15. Papa V, Gliozzo B, Clark GM, McGuire WL, Moore D, Fujita-Yamaguchi Y, Vigneri R, Goldfine ID, Pezzino V. 1993. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Res*. 53:3736-40.
16. Peyrat JP, Bonnetterre J, Vennin PH, Jammes H, Beuscart R, Hecquet B, Djiane J, Lefebvre J, Demaille A. 1990. Insulin-like growth factor 1 receptors (IGF1-R) and IGF1 in human breast tumors. *J. Steroid Biochem. Mol. Biol*. 37:823-7.
17. Freiss G, Rochefort H, Vignon F. 1990. Mechanism of 4-hydroxytamoxifen anti-growth factor activity in breast cancer cells: alterations of growth factor receptor binding sites and tyrosine kinase activity. *Biochem. Biophys. Res. Commun*. 173:919-26.

18. Kurose H, Yamaoka K, Okada S, Nakajima S, and Seino Y. 1990. 1,25-Dihydroxyvitamin D₃ [1,25-(OH)₂D₃] increases insulin-like growth factor (IGF-I) receptors in clonal osteoblastic cells. Study on interaction of IGF-I and 1,25-(OH)₂D₃. *Endocrinology*. 126:2088-94.
19. Chomczynski P, and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-9.

Figure 1

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

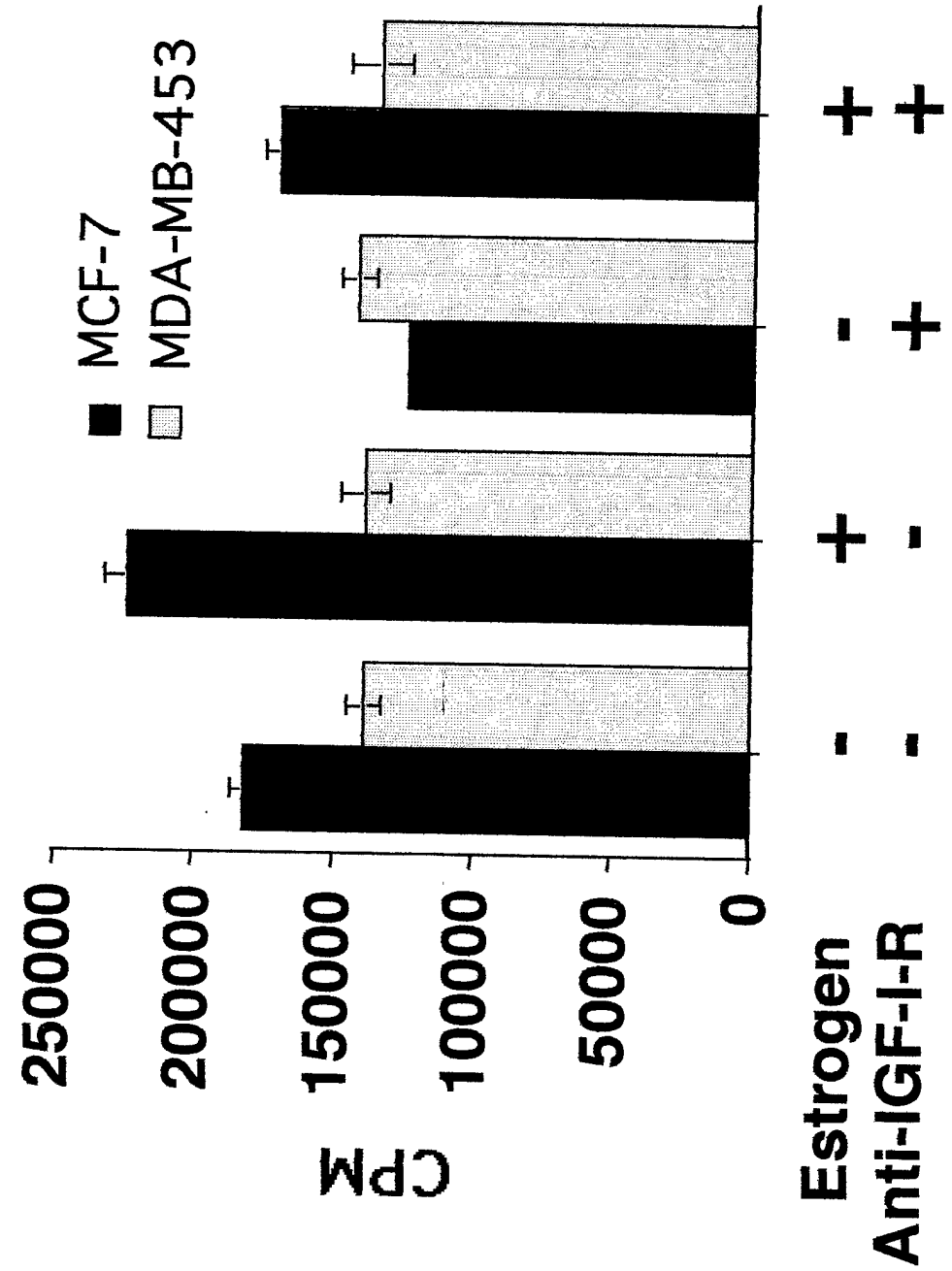
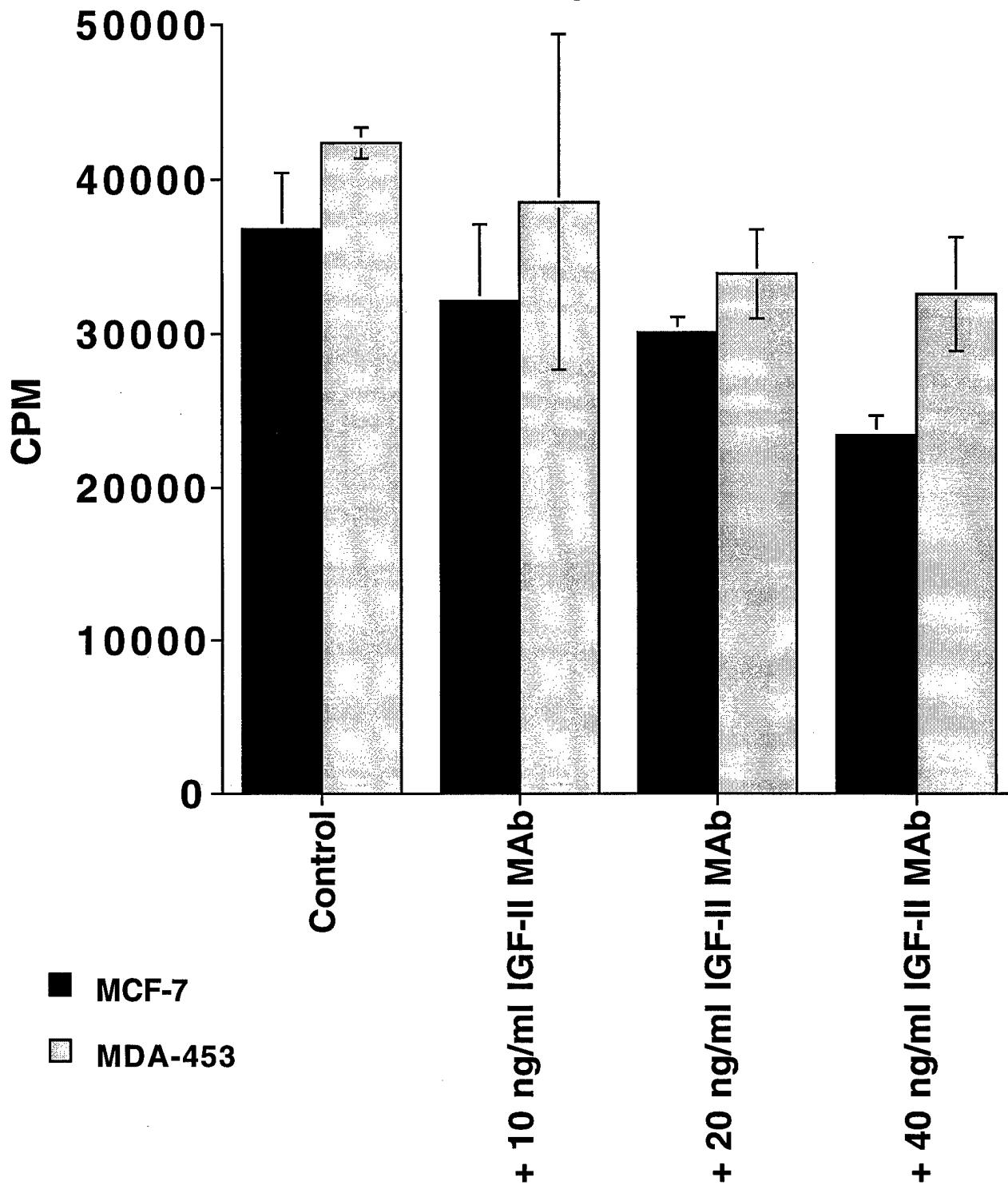


Figure 2

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



Effects of Anti-IGF-II Antibody in the Presence/Absence of Estrogen

Figure 3

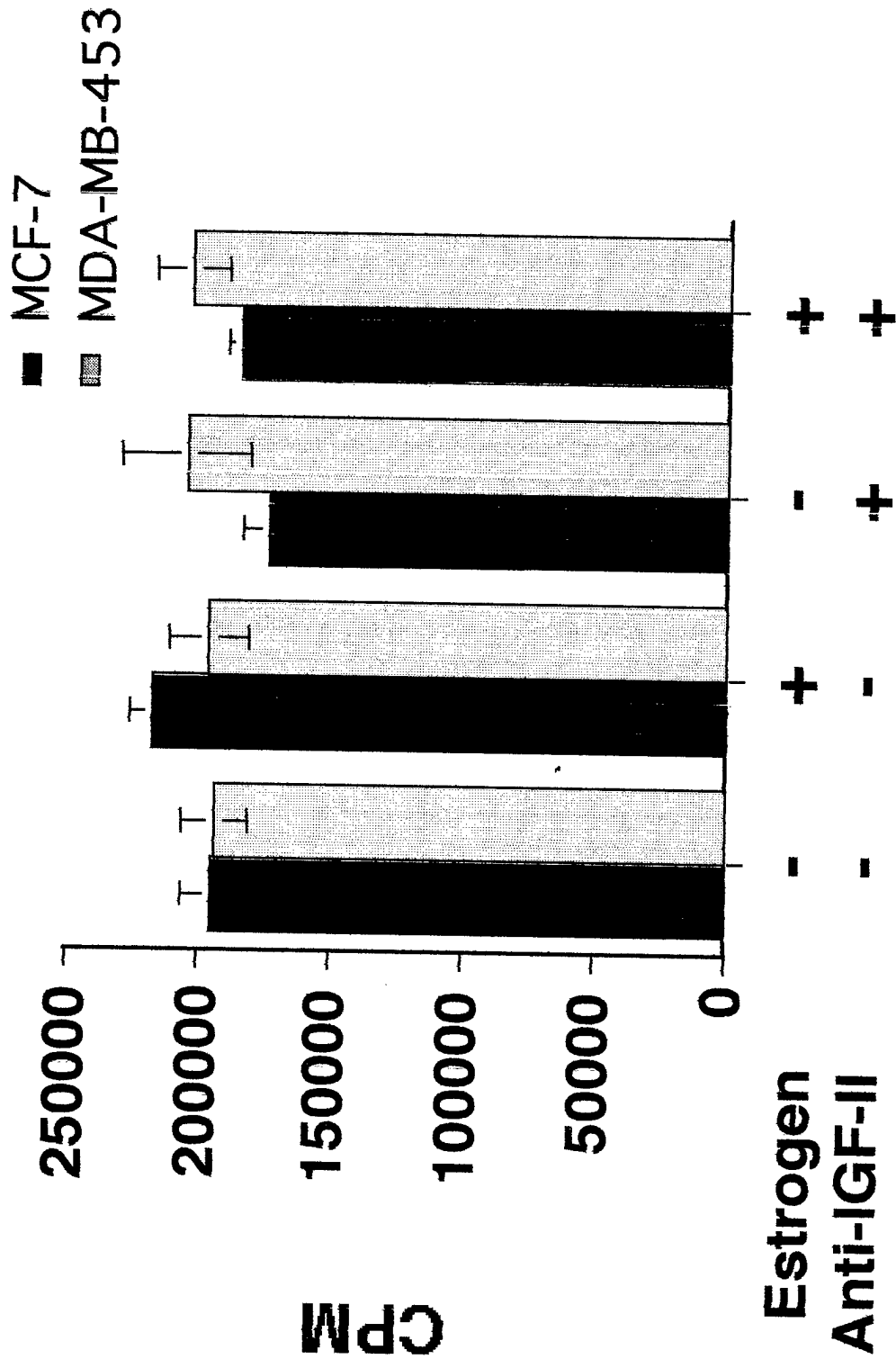


Figure 4b

Effects of Anti-IGF-II MAb on MDA-MB-453 in the Presence of E2, and 10^{-11} or 10^{-8} M $1,25(\text{OH})_2\text{D}_3$

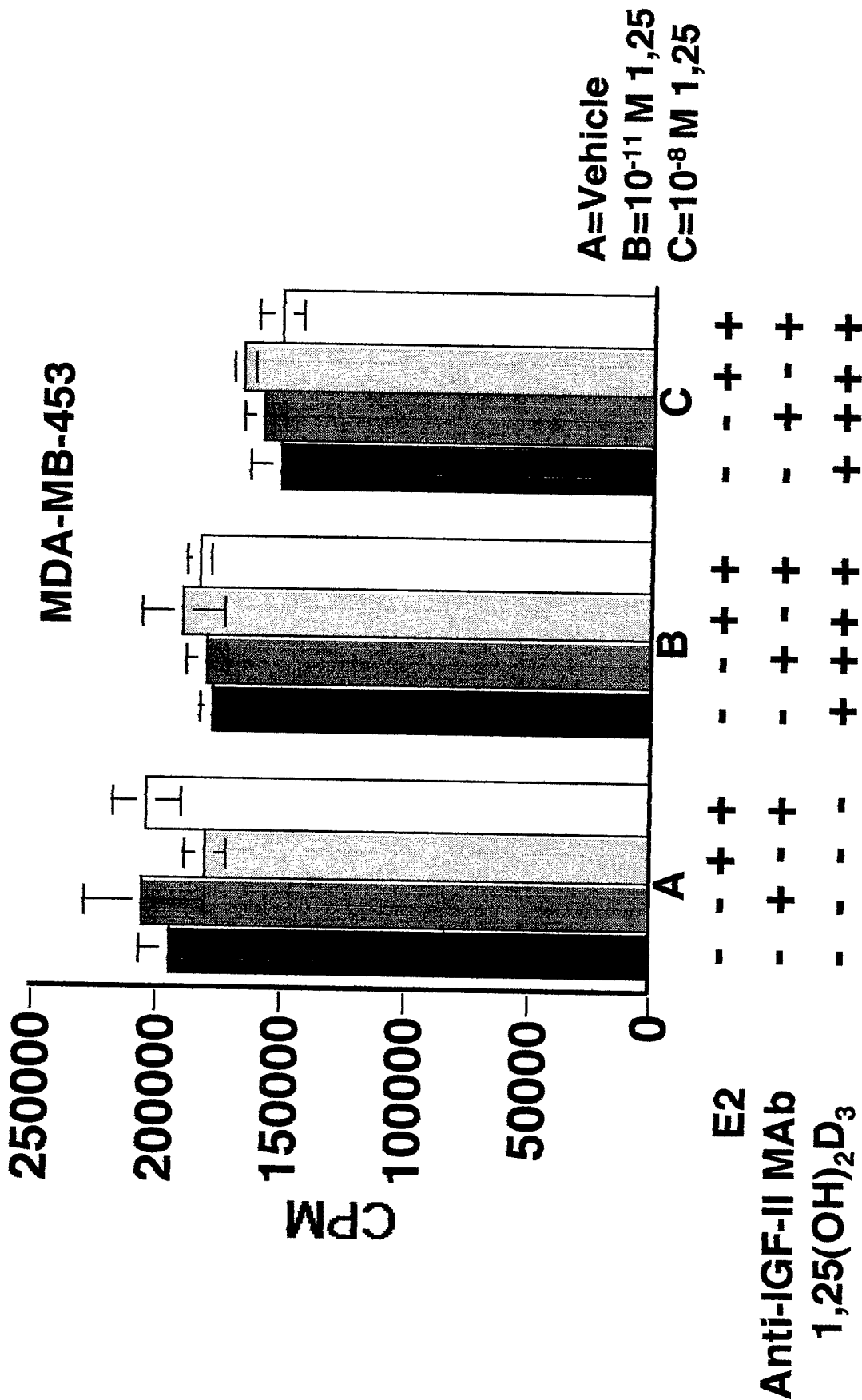


Figure 5a

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

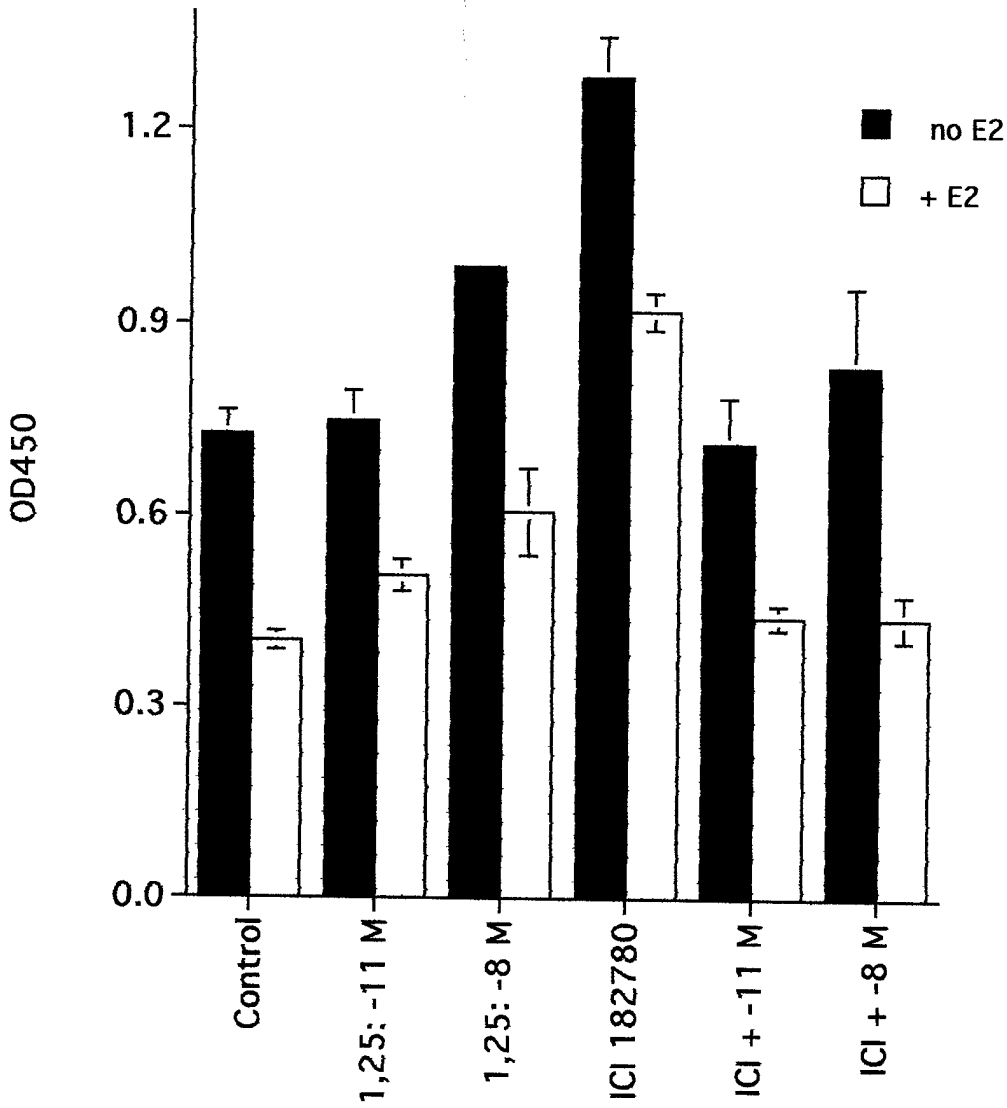


Figure 5b

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

