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13. ABSTRACT *(Maximum 200 words)*

We have observed that $1,25(OH)_2D_3$ and its analogs have a biphasic effect on the proliferation of estrogen receptor positive (ER+) breast cancer cell lines (BT-474, MCF-7) in that low concentrations proliferation, whereas the antiproliferative effects are not seen until concentrations above 10^{-9}M are employed. In contrast the ER- cell line, MDA-MB-453, is much more sensitive to the antiproliferative actions of $1,25(OH)_2D_3$ and the analogs. If the ER+ cell lines are incubated in the absence of estrogen or in the presence of the estrogen antagonist ICI 164384, the potency of $1,25(OH)_2D$ is markedly enhanced (by 2 to 3 orders of magnitude) with respect to its antiproliferative effects, essentially converting the response of these cells to that seen with MDA-MB-453 cells. Since estrogen appears to exert its proliferative actions on breast cancer cells in part by activating the insulin like growth factor (IGF) pathway, we evaluated whether $1,25(OH)_2D_3$ did likewise and if so whether estrogen modulated this action. Our studies demonstrated that $1,25(OH)_2D_3$ enhanced the autophosphorylation of the IGF-1 receptor (IGF-1R) in the presence of estrogen in a manner blocked by a specific estrogen antagonist. Blocking the ability of endogenously produced IGF-II with a specific antibody also blocked the proproliferative effects of estrogen plus low concentrations of $1,25(OH)_2D$. Thus, we hypothesize that estrogen blocks the antiproliferative action of $1,25(OH)_2D_3$ on breast cancer while promoting its proproliferative effects by regulating the IGF activated mitogenic pathway mediating the proliferative response. This interaction between two naturally occurring hormones, estrogen and $1,25(OH)_2D_3$, clearly has profound implications both for the etiology of breast cancer and for its treatment.

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INTRODUCTION

Breast cancer is the second leading cause of cancer deaths among females and a leading cause of death of all middle age women in the United States. Epidemiologic evidence suggests a role for vitamin D deficiency in the development of breast cancer. Moreover, vitamin D receptors have been found in most breast cancers, and their presence appears to be a favorable prognostic sign. While much clinical focus has been given to the vitamin A related retinoids, little is known about the role for vitamin D in the development of breast cancer although vitamin D and its active metabolites and analogs represent a class of compounds which at the molecular and cellular level have strong similarities to the vitamin A metabolites and analogs. With the development of potent but non hypercalcemic analogs of vitamin D, the potential role of vitamin D in the etiology and treatment of breast cancer can now be tested both in vitro and in vivo.

Nonclassic actions of vitamin D. In the past decade, a number of tissues have been found to contain receptors for the active vitamin D metabolite, $1,25(OH)_2D$, and to respond to this hormone with a change in function. The classic target tissues, bone, kidney, and intestine, responsible for maintaining bone mineral homeostasis in response to vitamin D and its metabolites are now only part of a list which includes several dozen tissues including various elements of the hematopoietic and immune system, cardiac, skeletal, and smooth muscle, brain, liver, breast, endothelium, skin (keratinocytes, melanocytes, and fibroblasts), and endocrine glands such as the pituitary, parathyroid gland, pancreatic islets (beta cells), adrenal cortex and medulla, thyroid, ovary and testis. Furthermore, malignancies developing within these tissues may also contain vitamin D receptors (VDR) and be expected to respond to $1,25(OH)_2D$. The responses of these tissues to $1,25(OH)_2D$ are as varied as the tissues themselves. $1,25(OH)_2D$ regulates hormone production and secretion including insulin from the pancreas, prolactin from the pituitary, and parathyroid hormone (PTH) from the parathyroid gland just as it regulates cytokine production and secretion such as interleukin-2 (IL-2) from the lymphocyte and tumor necrosis factor (TNF) from the monocyte. Myocardial contractility and vascular tone are modulated by $1,25(OH)_2D$ as is liver regeneration. $1,25(OH)_2D$ reduces the rate of proliferation of many cell lines including normal keratinocytes, fibroblasts, lymphocytes, and thymocytes as well as abnormal cells of mammary, skeletal, intestinal, lymphatic and myeloid origin. Differentiation of numerous normal cell types including keratinocytes, lymphocytes, hematopoietic cells, intestinal epithelial cells, osteoblasts, and osteoclasts as well as abnormal cells of the same lineage is enhanced by $1,25(OH)_2D$. Thus, the potential for manipulating a vast array of physiologic and pathologic processes with vitamin D related compounds is enormous.

Vitamin D analogs. The major problem facing the clinician desiring to manipulate any one of these newly recognized actions of vitamin D in vivo is that $1,25(OH)_2D$ is likely to require higher than physiologic doses to be effective and will not be selective at such doses. Thus, to use $1,25(OH)_2D$ to modulate the growth of a tumor is to risk complications associated with hypercalcemia and hypercalciuria. Developing analogs of $1,25(OH)_2D$ to improve the selectivity and confer a lower risk: benefit ratio has become a major effort by several pharmaceutical firms, and the early results look quite promising. In this proposal we will compare one of these analogs, EB1089, with $1,25(OH)_2D$ in vitro to parallel our studies in vivo (not part of this proposal) with this analog.

Application to breast cancer. Garland et al. (1,2) reviewed evidence correlating calcium and vitamin D with colon and breast cancer. Of 15 cancers evaluated, only these two showed a negative correlation between cancer incidence and the ambient ultraviolet (UV) light intensity when data from 87 locations throughout the United States were compiled. Similar data were

obtained from locations within what was formerly the USSR UV light exposure is suggested as a measure of cutaneous vitamin D production (3). The high frequency (90%) of breast cancers containing the vitamin D receptor (VDR) make the epidemiologic link between such cancers and vitamin D an especially attractive one for further investigation (4).

Eisman et al. (5) detected VDR in breast cancer lines over a decade ago. As mentioned above, this is not an artifact of culture in that the majority of breast tumors biopsied in vivo have been found to contain VDR. In general, $1,25(OH)_2D$ inhibits the proliferation and stimulates the differentiation of a number of breast cancer lines in vitro (5-10), although as will be discussed further below, this antiproliferative action is profoundly influenced by estrogen. Furthermore, $1,25(OH)_2D$ and its analogs have been shown to decrease tumor size, number, or lethality when given in vivo to animals in which the tumors were chemically induced or grafted (11-14). Although a dose response relationship between $1,25(OH)_2D_3$ and its antiproliferative effect has been demonstrated in vivo and in vitro, the dose in vivo is restricted by toxicity, in particular hypercalcemia and hypercalciuria (11,12,15). Similar constraints limit the use of $1,25(OH)_2D_3$ in the treatment of malignancies in humans (16). However, a number of $1,25(OH)_2D_3$ analogs, 22-oxa calcitriol (OCT), calcipotriol (MC903), and EB1089 have recently been shown to inhibit the proliferation of human breast cells in vitro at doses comparable to or less than $1,25(OH)_2D_3$ and to cause less hypercalcemia than comparable doses of $1,25(OH)_2D_3$ when administered in vivo (11-14). Calcipotriol is already approved for topical use in psoriasis, and when used topically in a small pilot study appeared to have a modest effect on breast tumor nodules in patients with local extension or cutaneous metastases (17). Likewise, 16ene, 23yne calcitriol (16ene, 23yne CT), which had not been evaluated in breast cancer until our recent studies, has been shown in mice to be more effective than comparable doses of $1,25(OH)_2D_3$ in increasing survival after the injection of leukemic cells, and does so at doses which do not induce hypercalcemia (18). Thus, the availability of relatively non hypercalcemic analogs of $1,25(OH)_2D_3$ offers a promising means to permit in vivo studies of the etiology and treatment of breast cancer. Nevertheless, further enhancement of the antiproliferative potency of these analogs in vivo is an important goal which appears to be reachable based on recent studies from our laboratory and those of others regarding $1,25(OH)_2D_3$/estrogen interactions.

$1,25(OH)_2D$/estrogen interactions. $1,25(OH)_2D_3$, of course, is not the only hormonal regulator of breast cancer growth. Estrogens are well known promoters of breast cancer development, and antiestrogens like tamoxifen have been an important component of therapy in hormone responsive tumors. Abe-Hashimoto et al. (13) demonstrated that tamoxifen potentiated the ability of the $1,25(OH)_2D_3$ analog OCT to suppress breast cancer tumor growth in vivo. This observation has been confirmed in vitro with other $1,25(OH)_2D_3$ analogs and with other estrogen antagonists. Our own data show that the pure antiestrogen ICI 168384 potentiates the antiproliferative actions of $1,25(OH)_2D_3$ in estrogen receptor positive (ER+) cell lines by 2 to 3 orders of magnitude. Such observations indicate an interaction between estrogen and $1,25(OH)_2D$ that has importance not only for treatment of breast cancer but also for an understanding of its development in a hormonal milieu which may promote breast cancer cell growth.

BODY

1.Development of serum free culture conditions for breast cancer cells.

Because serum contains vitamin D metabolites and their binding protein (vitamin D binding protein or DBP), IGFs and their binding proteins, growth hormone (GH), and a wide variety of

other growth factors which may vary from lot to lot, we elected to develop a serum free cell culture system that would permit the study of breast cancer cell lines in a more controlled fashion. We observed that the cell lines MCF-7, BT-474, and MDA-MB-453 could be adapted to serum free media supplemented only with ITS+ (insulin, transferrin, selenium). All of our studies have been performed in this medium. In general 10^{-10}M E2 was included in the media of the ER+ cell lines, although the ER+ cell lines studies grew well without E2 permitting interactions between E2 and $1,25(OH)_2D$ to be studied.

2. Determine the VDR levels in these breast cell lines.

Northern analysis of the RNA extracted from the three cell lines was performed using a cDNA probe for the VDR mRNA. The results are shown in figure 1. All three cell lines contain the mRNA for VDR at comparable levels. We then analyzed the cells for functional VDR using ligand binding methodology and analyzing the data by Scatchard analysis. These results are shown in figure 2. In this experiment we compared the affinity of the different analogs for the VDR to that of $1,25(OH)_2D_3$ itself. As shown, all cell lines contained a functional VDR, with highest levels in the MDA-MB-453 cell line. The analogs studied in this project all have high affinity for the VDR, generally within the same order of magnitude as $1,25(OH)_2D_3$ itself.

3. Effect of $1,25(OH)_2D$ and its analogs on growth of MCF-7, BT-474, and MDA-MB-453.

Dose response curves to $1,25(OH)_2D$ and its analogs 16ene calcitriol (16ene), 16ene,23yne calcitriol (16ene,23yne), EB1089, and 22-oxa calcitriol (OCT) for each of the three cell lines are shown in figure 3. The three cell lines were chosen to represent high (MCF-7), low (BT-474), and no (MDA-MB-453) ER levels. In the two ER+ cell lines and in the presence of E2, $1,25(OH)_2D$ and its analogs tended to stimulate proliferation at doses below 10^{-9}M, with no clear antiproliferative effects seen until doses above 10^{-8}M were employed. The extent of this proproliferative effect and the concentration of $1,25(OH)_2D_3$ or analog at which it was maximal varied somewhat from experiment to experiment and analog to analog (eg. EB1089 did not show a proproliferative effect in BT-474 cells in the experiment shown in figure 3, but had such an effect in other experiments). In contrast, although some of the analogs stimulated the ER- cell line MDA-MB-453 at 10^{-12}M, concentrations above this were antiproliferative. Individual analogs differed somewhat in potency of these effects, but the trends were comparable. Efforts to induce differentiation of these cell lines by $1,25(OH)_2D_3$ and its analogs were not successful.

4. Assess the synergy between antiestrogens and the calcitriol analogs on proliferation.

The antiestrogen ICI 164384 also had an antiproliferative effect on the breast cancer cell lines (figure 4), being more effective on the ER+ cell lines as expected, but it also inhibited the ER- cell line MDA-MB-453. When ICI 164384 was combined with $1,25(OH)_2D$ at a concentration of the ICI 164384 which by itself had little or no antiproliferative activity, $1,25(OH)_2D$ now became a much more potent antiproliferative agent (figure 5). In particular, the response of BT-474 and MCF-7 to $1,25(OH)_2D$ in the presence of ICI 164384 resembled the response of MDA-MB-453 to $1,25(OH)_2D$ in the absence of ICI 164384 (figure 3). Similarly, if E2 was omitted from the medium, the antiproliferative actions of $1,25(OH)_2D$ on MCF-7 cells (ER+) were equivalent in potency as the antiproliferative actions of $1,25(OH)_2D$ on MDA-MB-453 cells (figure 6). These data suggest that estrogen through its ER blocks the antiproliferative actions of $1,25(OH)_2D$ and promotes its proproliferative actions. Thus in ER+ cells, the combination of estrogen and low doses of $1,25(OH)_2D$ have additive proproliferative effects.

To assess whether the ability of estrogen to promote the proliferative and block the antiproliferative actions of $1,25(OH)_2D$ was mediated through changes in IGF-II production and/or IGF-IR activation, we performed the following series of experiments. First we measured IGF-1 stimulated IGF-1R tyrosine phosphorylation following incubation of MCF-7 cells with 10^{-10} and 10^{-8}M $1,25(OH)_2D$ in the presence and absence of 10^{-8}M ICI 164384. In this experiment 10^{-10}M estrogen was present, but insulin was excluded from the medium. The results are found in figure 7. In the presence of estrogen, $1,25(OH)_2D$ increased IGF-1 stimulated IGF-1R tyrosine phosphorylation by 64%. ICI 164384 alone also had a slight stimulatory effect (not significant in this experiment), but blocked the increase seen with $1,25(OH)_2D$. Given the likely role that IGF-1R activation plays in proliferation, these data support the hypothesis that estrogen enhances the proliferative actions of $1,25(OH)_2D$ at the level of the IGF-1R. Since the ability of $1,25(OH)_2D$ to stimulate IGF-1R activation increased with increasing dose, it would appear that the antiproliferative actions of higher doses of $1,25(OH)_2D$ are not mediated through a reduction in IGF-1R activation. We then assessed whether the interaction between E2 and $1,25(OH)_2D$ on proliferation could be blocked by blocking IGF action with an antibody to IGF-II (figure 8). As shown in figure 8A, the proliferative response to E2 and 10^{-11}M $1,25(OH)_2D$ in MCF-7 cells (ER+) is blocked much more effectively by anti IGF-II than is the response to E2 alone. $1,25(OH)_2D$ in the absence of E2 is not proproliferative, and the response to $1,25(OH)_2D$ alone is minimally effected by anti IGF-II. MDA-MB-453 cells (ER-) (figure 8B) do not show the synergism between E2 and $1,25(OH)_2D$, and anti IGF-II has little effect on the proliferation of these cells. Thus, our results point to the importance of the IGF pathway in mediating the interaction between E2 and $1,25(OH)_2D$ in ER+ cells.

5. Determine the ability of the calcitriol analogs to suppress the growth of breast cancer cells inoculated into nude mice. MCF-7 cells were prepared in a growth factor matrix gel and inoculated at a dose of 2.5 million cells/mouse into nude mice which had received a subcutaneous estrogen pellet prior to inoculation. Tumor growth occurred in all mice. Initial experiments waited until the tumor size was $1cm^3$ before administration of the vitamin D analog. Subsequent experiments were performed by administering the drug after the tumor had reached $100mm^3$ or the drug was begun immediately after implantation. EB1089 was the analog studied in all experiments. Initial experiments compared oral administration to intraperitoneal administration. The initial formulation was in proplene glycol/ethanol for oral administration and peanut oil/ethanol for intraperitoneal administration. The intraperitoneal administration caused substantial toxicity, with high mortality and intraabdominal adhesions thought to be a result of the vehicle. Doses up to 0.8ug/kg orally in most experiments were tolerated with limited increases in serum calcium, although higher doses resulted in a dose dependent increase in serum calcium. Regardless of dose, no consistent effect on tumor growth was seen in these experiments. To evaluate whether the estrogen pellets were obscuring the effect of EB1089, we repeated the experiment, removing the pellet from half of the animals after 28d. Removal of the estrogen pellet caused regression of the tumor, but animals receiving EB1089 did not show a faster regression than those receiving vehicle.

6. Assess the synergy between antiestrogen and calcitriol analogs on tumor growth. We used the same approach as for task 5, namely nude mice with estrogen pellets inoculated with MCF-7 cells. The antiestrogen was ICI 182780, a specific antiestrogen previously shown by our collaborator Dr Wakeling to block breast cancer growth in mice. We had performed preliminary experiments in vitro and shown that MCF-7 cells were quite sensitive to the antiproliferative actions of ICI 182780, a more potent antiestrogen than ICI 164384 in those experiments, and that ICI 182780 and EB1089 had synergistic antiproliferative actions in vitro. Experiments were performed evaluating the effect of EB1089 alone, ICI 182780 alone, and the two together. The

results were quite disappointing in that neither drug alone or in combination was effective in preventing tumor growth. Only one dose of ICI 182780 was used in these experiments (the dose recommended by Dr. Wakeling), and the high cost of doing these in vivo experiments relative to the limited budget awarded necessitated truncation of this approach before antiproliferative doses of these drugs could be determined in this model.

CONCLUSIONS

1. The breast cancer cell lines BT474, MCF-7, and MDA-MB-453 vary in their content of estrogen receptor, but all have the vitamin D receptor capable of binding $1,25(OH)_2D_3$ and the analogs used in this study with high affinity.

2. In the presence of estrogen, $1,25(OH)_2D_3$ and its analogs tend to have a biphasic effect on the proliferation of ER+ breast cancer cells. Low and physiologic concentrations of $1,25(OH)_2D_3$ and the analogs actually appear to promote growth. However, when the cell has no ER or the ER is blocked with an antagonist the antiproliferative actions of $1,25(OH)_2D_3$ and the analogs are readily seen. Thus effective therapy of breast cancer with the $1,25(OH)_2D_3$ analogs is likely to involve combinations with an antiestrogen, although we were unable to prove this concept in nude mice inoculated with MCF-7 breast cancer cells.

3. The proproliferative actions of $1,25(OH)_2D_3$ and the analogs in the presence of estrogen raise the question of mechanism. Our data lead us to suggest that the insulin like growth factor pathway is involved in that $1,25(OH)_2D_3$ stimulates the autophosphorylation, and so activation, of the IGF-1 receptor and that inhibiting the endogenous ligand of the IGF-1R, namely IGF-II, with an anti IGF-II antibody blocks the proliferative effects of estrogen alone or in combination with low doses of $1,25(OH)_2D$.

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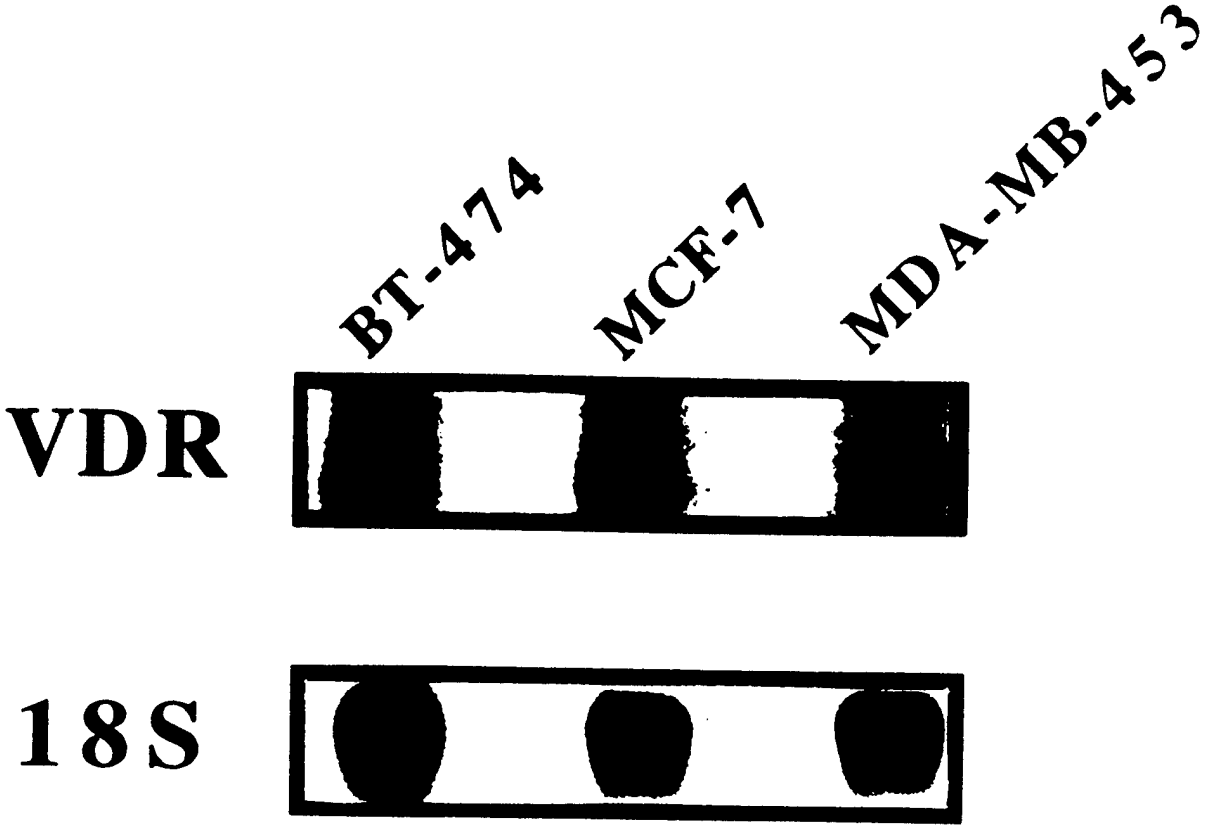
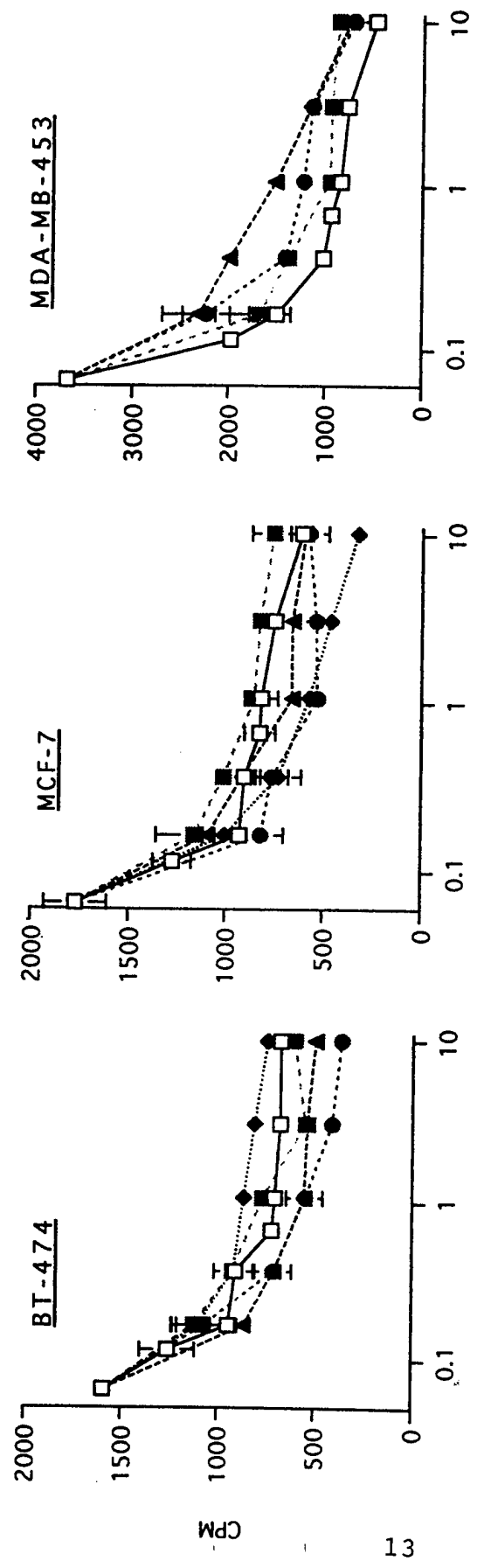


VDR mRNA LEVELS IN BREAST CANCER CELLS

VDR mRNA levels were measured by Northern blot analysis, and normalized to 18 S RNA.

Figure 1



The displacement of [3H]-1,25(OH)2D3 from the VDR by various analogs from the VDR by 1,25$(OH)_2D_3$ (□), 16ene (◆), 16ene,23yne (●), EB1089 (▲), and OCT (■) is shown for the three breast cancer cell lines. Data are expressed as mean cpm ± sd of triplicates.

Figure 2

THE EFFECTS OF $1,25(OH)_2D_3$ AND ITS ANALOGS ON PROLIFERATION OF BT-474, MCF-7, AND MDA-MB-453 CELLS

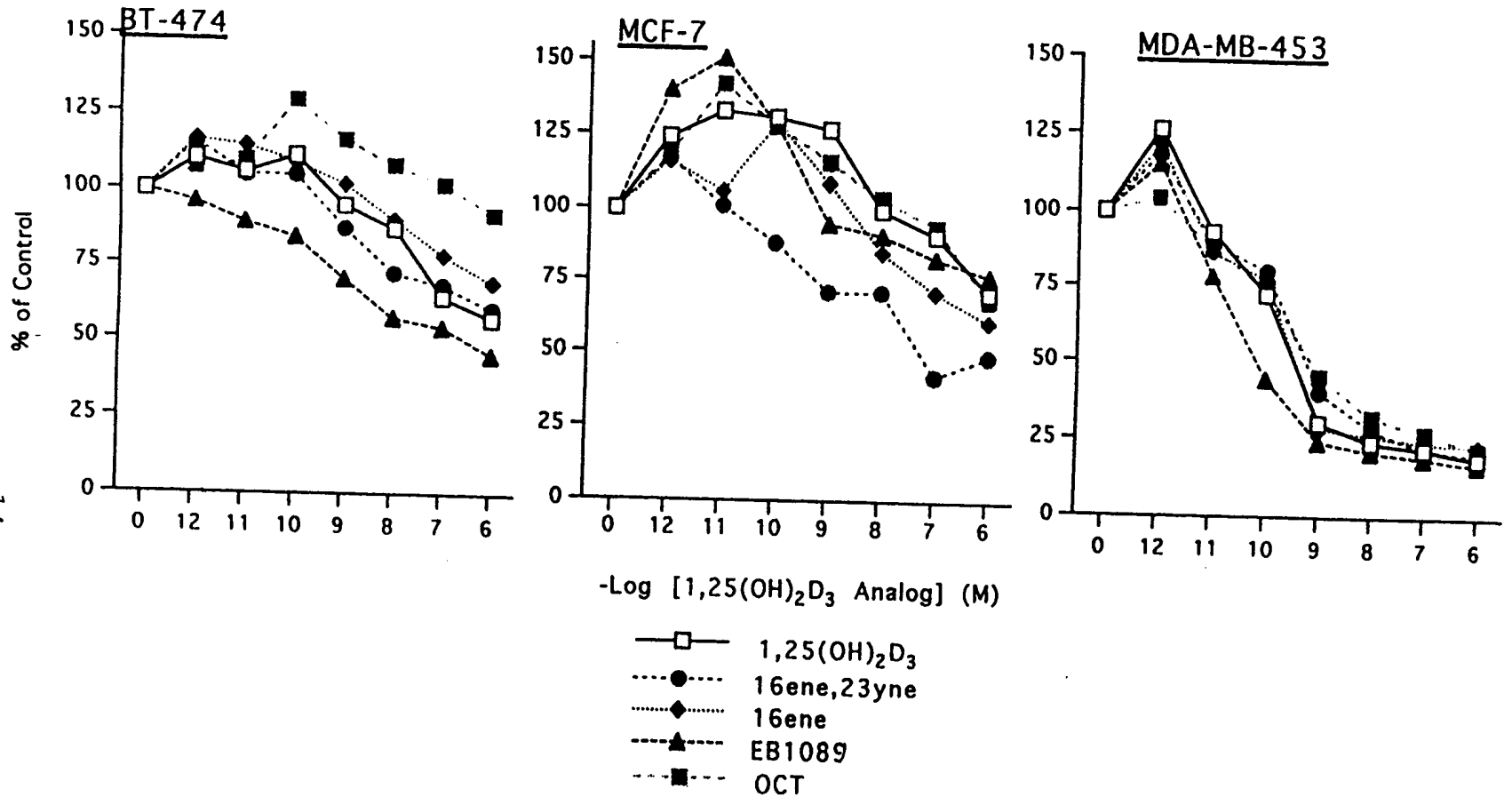


Fig. 3: Cells were treated with $1,25(OH)_2D_3$, 16ene, 16ene,23yne, EB1089 or OCT at the indicated concentrations for 3 d, and proliferation was measured using [^{3}H]-thymidine incorporation. Data are expressed as percentage of the control values (vehicle-treated) and represent the mean of triplicates. Similar results were obtained in 2 separate experiments.

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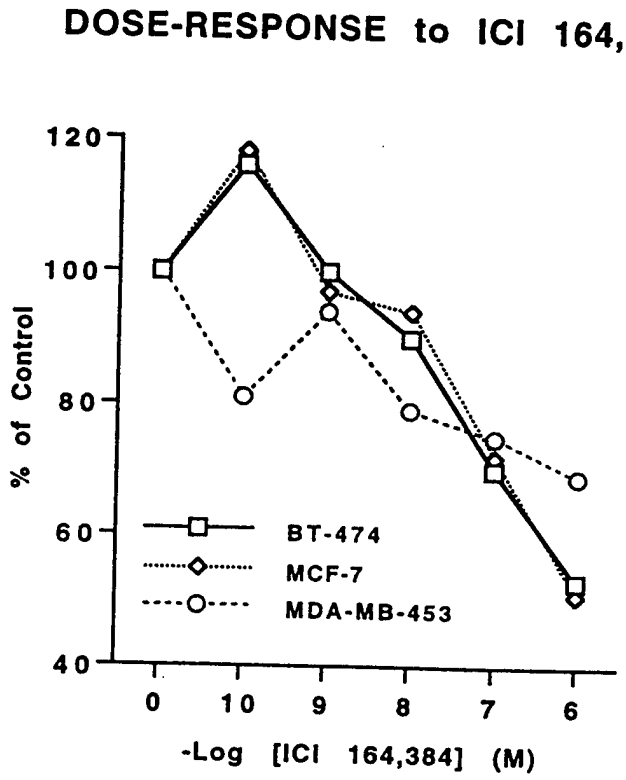

Fig. 4. Cells were treated with the indicated concentrations of ICI 164,384 for 3 d, then proliferation was measured using [H]-thymidine. Data are expressed as percentage of the control values, and represent the mean of triplicates. Similar results were obtained in 4 separate experiments.

THE EFFECTS OF $1,25(OH)_2D_3$ IN COMBINATION WITH 10^{-8} M ICI164,384
ON PROLIFERATION OF BREAST CANCER CELLS

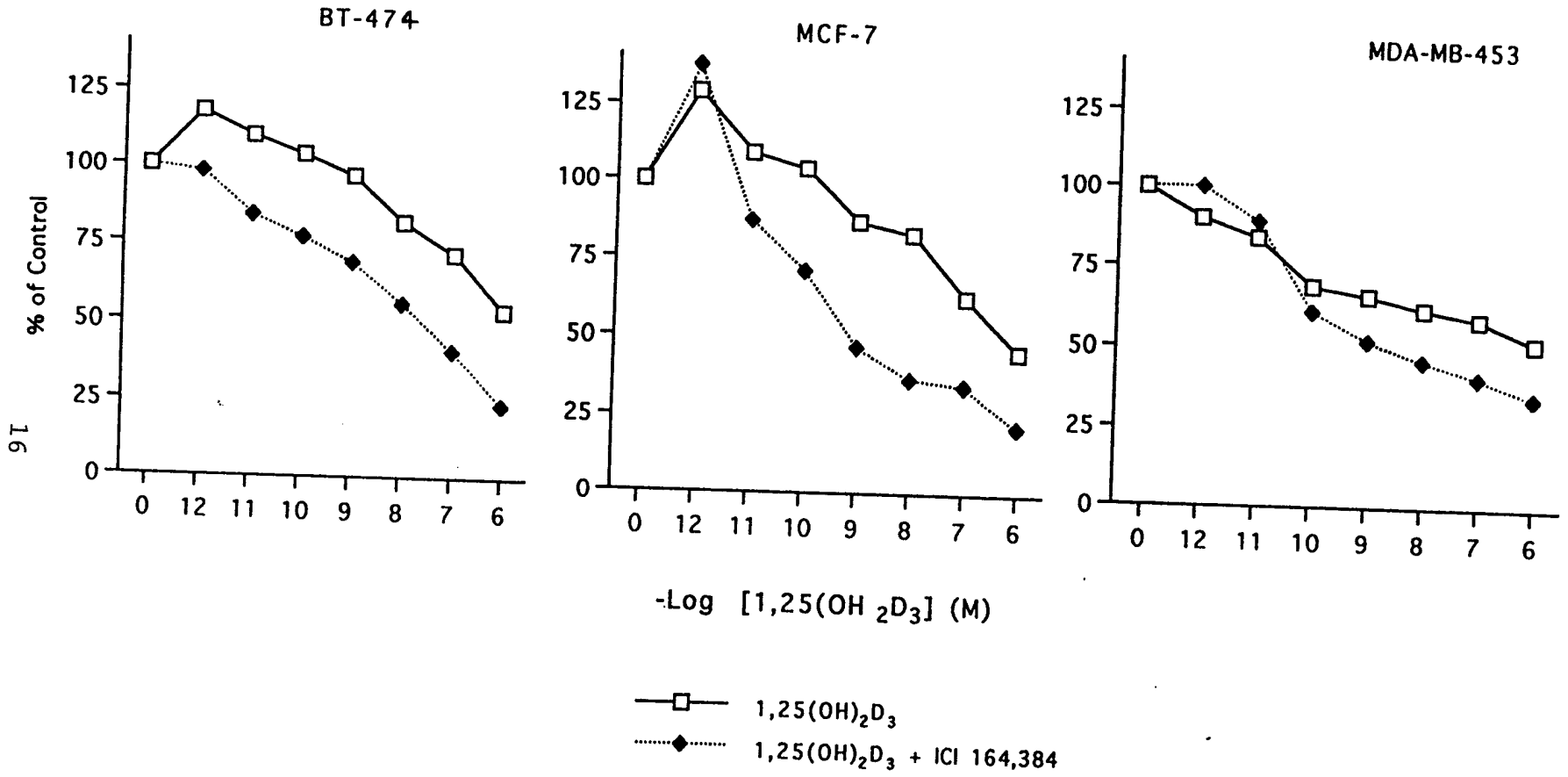

Fig. 5: Cells were treated with $1,25(OH)_2D_3$ at the indicated concentrations, or $1,25(OH)_2D_3$ + 10^{-8} M ICI 164,384 for 3 d. Proliferation was measured using [^{3}H]-thymidine. Data are expressed as percentage of control values, and represent the mean of triplicates. Similar results were obtained in 2 separate experiments.

PI: Daniel D. Bikle, M.D.,Ph.D.

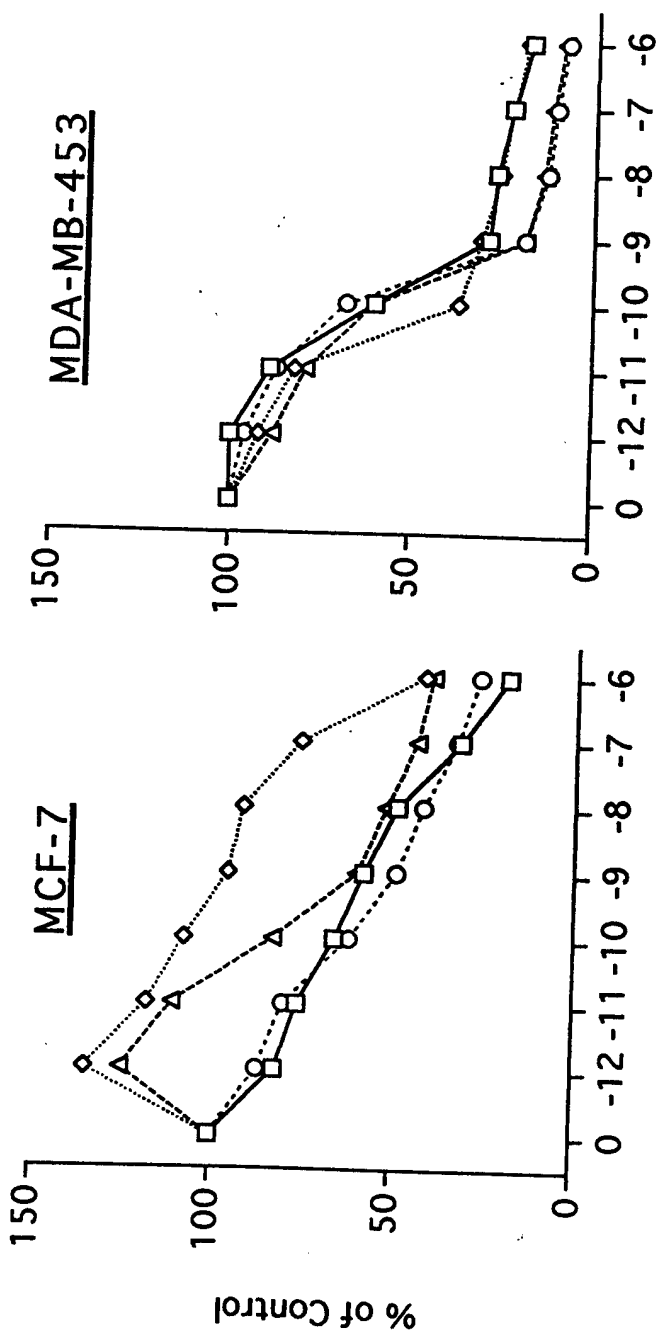

Figure 6: Breast cancer cells were incubated in the presence of 1,25(OH)$_2$D (□,◊) or EB1089 (○,△) in the presence (◊,△) or absence (□,○) of E_2. Each point is the mean of triplicate determination, the SD of which was < 8% of the mean.

Effect of E_2 and $1,25(OH)_2D_3$ on IGF-I-Stimulated IGF-I-R Tyrosine Phosphorylation in MCF-7 Cells

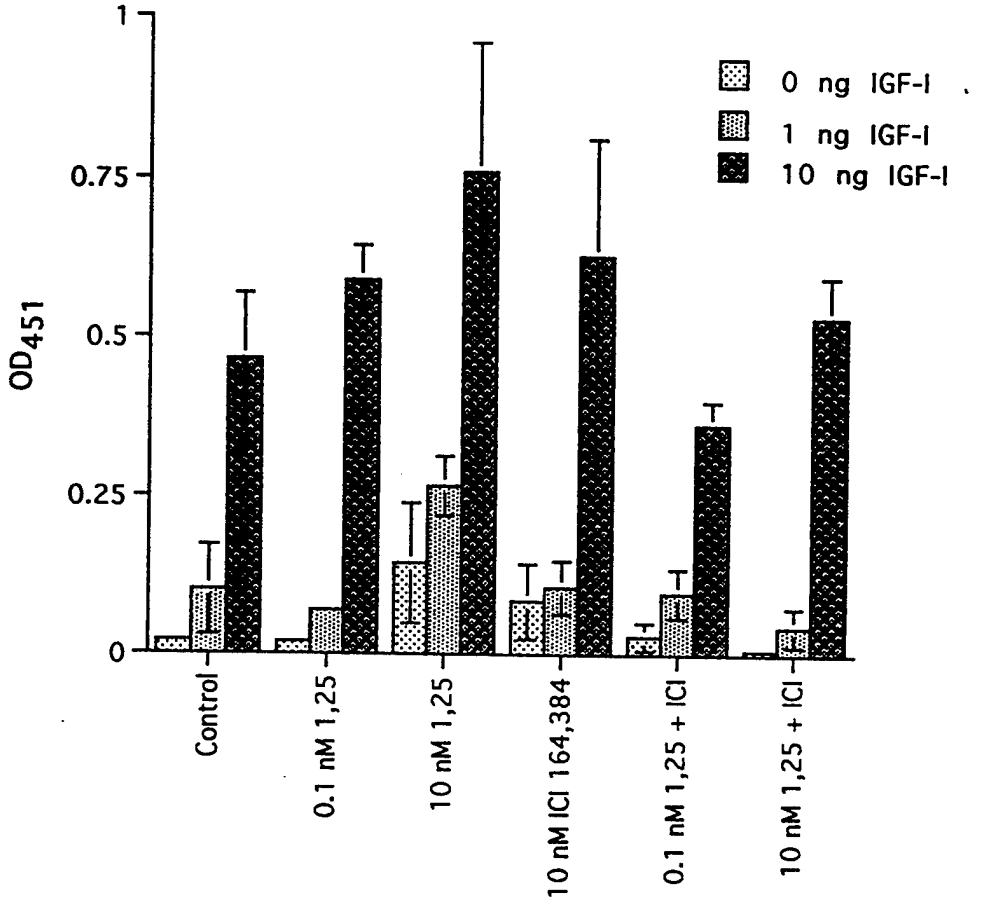

Figure 7 : Data are expressed as mean ± SD of duplicate OD_{451} determinations. The cells were incubated in serum-free, insulin-free medium containing 10^{-10} M E_2 and the indicated concentrations of $1,25(OH)_2D_3$ and ICI 164,384. The assay utilized ELISA with anti-IGF-I-R (1° MAb α-IR3), and biotinylated anti-phosphotyrosine (2° MAb), using streptavidin-linked HRP as detection agent.

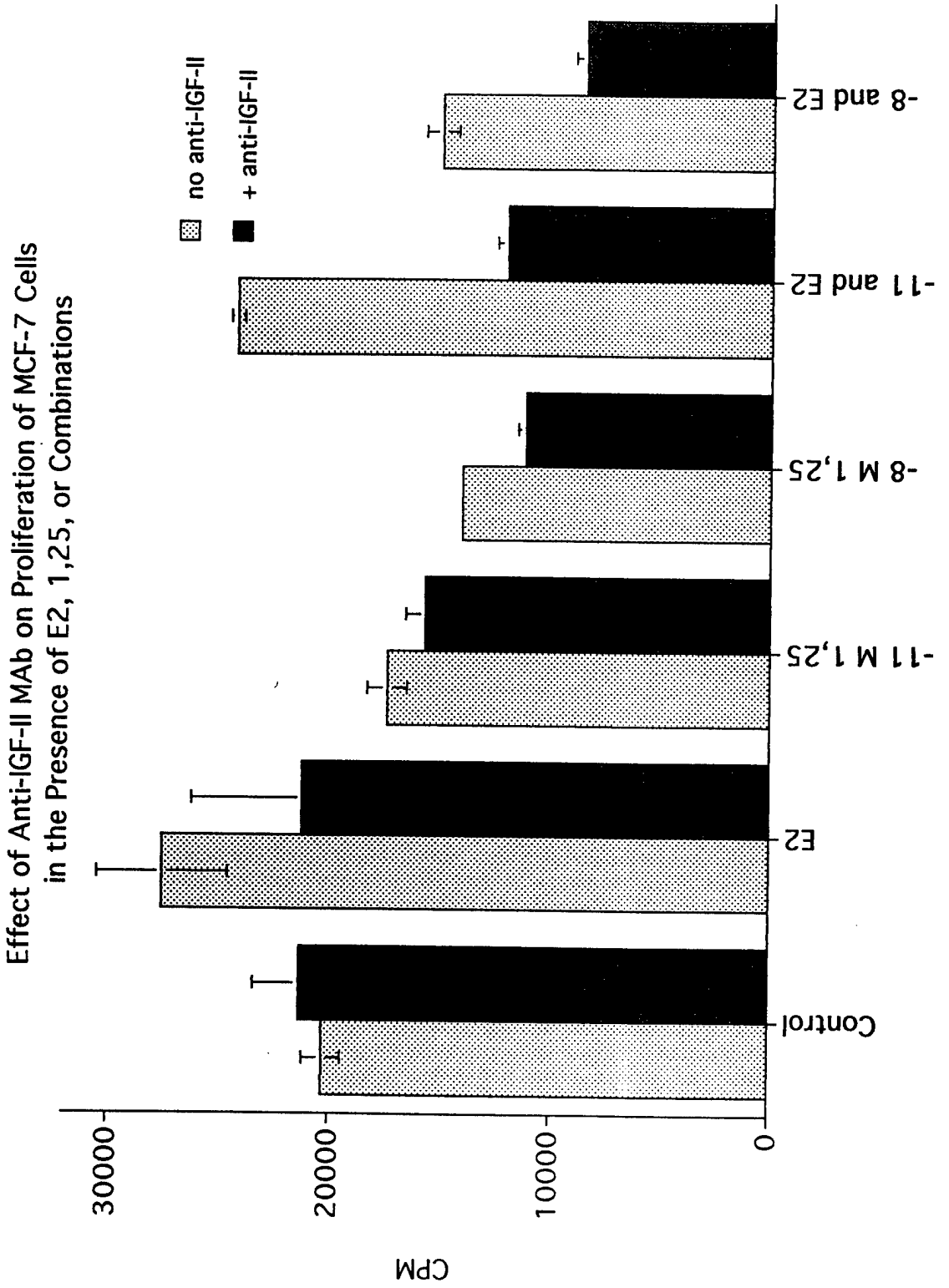

Fig. 8a Effect of anti-IGF-II monoclonal antibody on proliferation of MCF-7 cells. Cells were treated with $1,25(OH)_2D_3$ at the indicated concentrations, E_2 at 10^{-10} M, or combinations of both for 1 day. Proliferation was measured using [^{3}H]thymidine incorporation. Data are expressed as mean cpm of triplicates ± standard deviation.

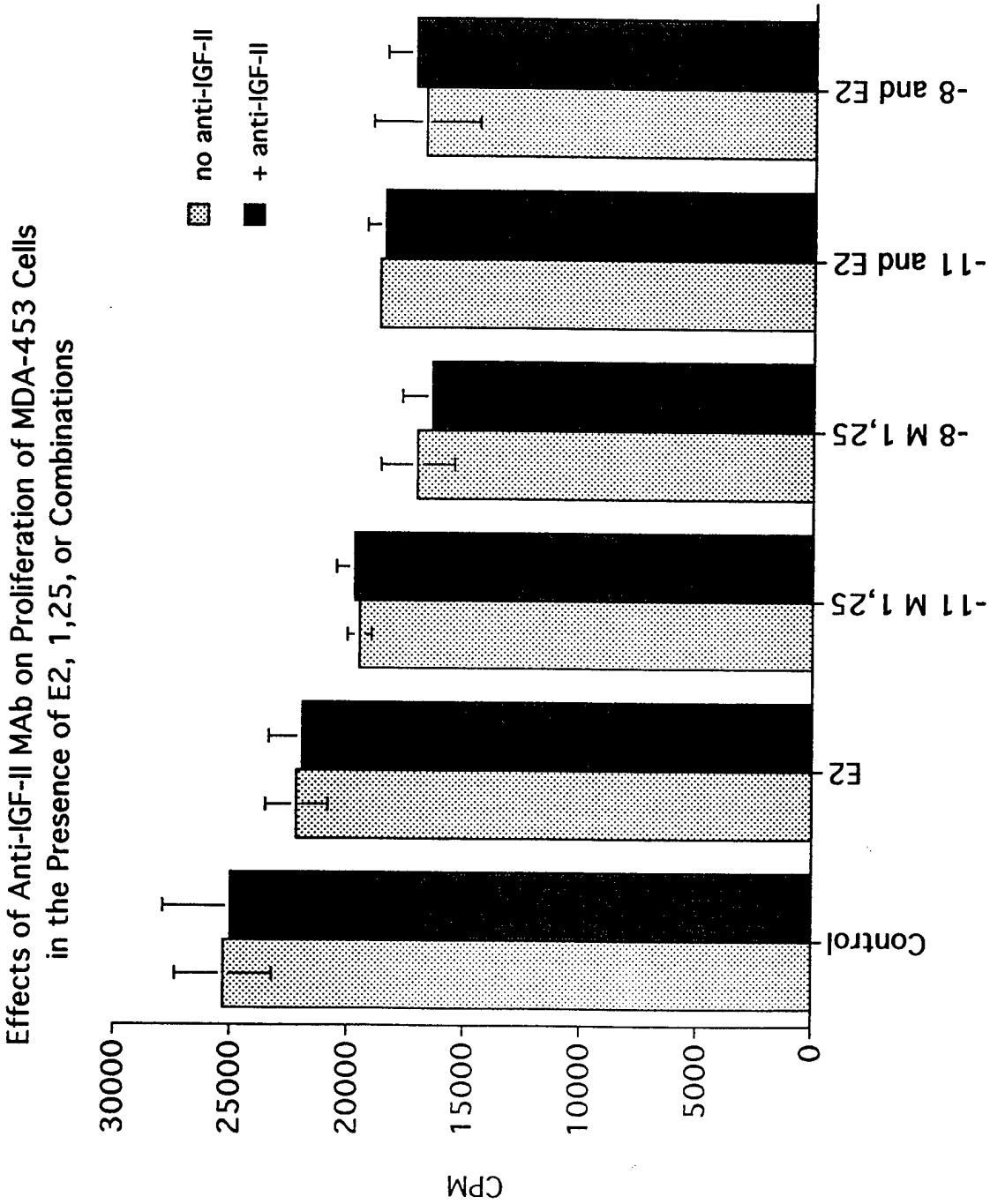

Fig. 8b Effect of anti-IGF-II monoclonal antibody on proliferation of MDA-MB-453 cells. Cells were treated with 1,25$(OH)_2D_3$ at the indicated concentrations, E_2 at 10^{-10} M, or combinations of both for 1 day. Proliferation was measured using [^{3}H]thymidine incorporation. Data are expressed as mean cpm of triplicates ± standard deviation.