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13. ABSTRACT *(Maximum 200 words)*
The T47D human breast cancer cell line was grown in triplicate for 7 days in the presence or absence of the following hormonal treatments: estradiol (10^{-6}-10^{-9}M), tamoxifen (10^{-7}M) or tamoxifen (10^{-7}-10^{-9}M) with 10^{-8}M estradiol, calcitriol (10^{-8}M), cortisone (10^{-7}-10^{-9}M), triiodothyronine (10^{-7}-10^{-9}M), progesterone (10^{-6}-10^{-10}M), testosterone (10^{-6}-10^{-10}M), retinoic acid (10^{-6}-10^{-10}M), or dexamethasone (10^{-7}-10^{-9}M). No consistent dose-related effect on the growth was observed. The following human cell lines were screened in triplicate for the effect of calcitriol (10^{-8} M) on cell growth: T47D, CRL-1500, CRL-1897, HTB-20, HTB-23, HTB-24, HTB-26, HTB-27, HTB-30, HTB-122, HTB-126, HTB-131. Again, no consistent effect on cell growth was observed. The presence of the vitamin D receptor was verified in T47D, HTB-20, and CRL1500 cells by PCR. To further investigate the action of more potent calcitriol analogues on cell growth, T47D, MCF-7, HTB-20, and HTB-131 cells were exposed to calcitriol, 22-oxacalcitriol, 16ene-calcitriol, and 16ene, 23 yne-calcitriol. Cellular proliferation was assessed in these experiments by ^{3}H-thymidine incorporation and all treatments were done in triplicate or quadruplicate. No antiproliferative effect of calcitriol or its analogues over a 6 log concentration range was observed in any cell line tested. Calcitriol does not appear to be a modulator of cell growth in cultured human breast cancer cells and the use of calcitriol or its analogues does not appear to be a promising approach to therapy of breast cancer.

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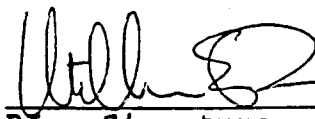

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INTRODUCTION

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$ or calcitriol), the active hormonal form of vitamin D, has been shown to have many more diverse biologic actions than its role in calcium and skeletal homeostasis. This hormone has been found to modulate hormone secretion, regulate immune system function, inhibit cellular replication, and induce differentiation (1-3). The vitamin D receptor (VDR) appears to be necessary for calcitriol to exert an antiproliferative effect on cell growth. This conclusion is based on three lines of evidence: 1) There is a correlation between cell growth rates and $1,25(OH)_2D_3$ receptor levels in vitro (4-7) and in vivo (8). 2) The antiproliferative effects of vitamin D metabolites correlate with their binding affinity for the VDR (9). 3) Cells that are resistant to the antiproliferative effects of calcitriol have reduced cellular uptake of $[^3H]$-$1,25(OH)_2D_3$ and decreased or absent vitamin D receptors (10-13). The antiproliferative effects of $1,25(OH)_2D_3$ correlate not only with the presence of the VDR but also with their concentration in target cells (14-16). Normal breast tissue has been shown to contain receptors for vitamin D (17-20). The VDR is also present in several established human breast cancer cell lines: T47D (21-23), MDA-MB-231 and ZR-75-1 (24), HsO578T (25), and MCF-7 (19,26,27).

Calcitriol inhibits the in vitro proliferation of several breast cancer cell lines: T47D (9,24), MCF-7 (13,15,24,28,29), HT-39 (15,28), BT20 (29), HS811 (13), ZR-75-1 (13, 24), and Hs05787 (25). In vivo, treatment of mice with a nitrosomethylurea-induced

mammary tumor with 1 alpha (OH) D_3 produced a significant inhibition of tumor progression (13). Treatment of 14 patients with locally advanced or cutaneous metastatic breast cancer with topical calcipotriol (another vitamin D analogue which is as active as calcitriol in inducing cell differentiation in vitro but has 100 times less effect of calcium metabolism in vivo) resulted in local responses in four patients (30). All four of the responsive tumors had measurable concentrations of vitamin D receptor. These in vitro and in vivo studies indicate that vitamin D_3 metabolites or analogues may be effective antitumor agents in the treatment of breast cancer.

While the mechanism(s) of calcitriol inhibition of cellular proliferation is (are) unknown, calcitriol regulates cellular oncogene expression in other cell types. The goals of this project were to determine the effects of calcitriol and various hormones, demonstrated to alter the concentration of the VDR in other cells, on the proliferation of human breast cancer cells in culture. Also anticipated to be studied was the effect of these hormones on the concentration of the VDR in breast cancer cells, on calcitriol inhibition of human breast cancer cell growth, and on oncogene expression.

The objectives of this project are:

OBJECTIVE 1: To determine the effects of various hormonal treatments on the concentration and affinity (Kd) of the vitamin D

receptor (VDR) and on the proliferation of human breast cancer cells. (estimated time to accomplish: months 1-5)

OBJECTIVE 2. To determine the effects of combinations of the active hormonal therapies identified by objective 1 on the VDR concentration and on proliferation of cultured breast cancer cells to determine if there is any synergy between the active hormonal agents. (estimated time to accomplish: months 6-7)

OBJECTIVE 3: To determine the effects of the hormonal treatments shown to increase the concentration of the VDR or those hormones that alter cellular proliferation, on calcitriol inhibition of human breast cancer cell growth. (Estimated time to accomplish: months 8-9)

OBJECTIVE 4: To determine the effects of those hormonal treatments active in either increasing the VDR or altering cellular proliferation on the expression of several protooncogenes that are overexpressed in human breast tumors. (Estimated time to accomplish: months 10-12)

The work on this project began on 1 September 1993 after Dr Jian Yu Feng, a postdoctoral fellow from China was hired. To determine the effects of various hormonal treatments on the proliferation of human breast cancer cells, MCF-7 human breast cancer cells were purchased and cultured in phenol red free Eagle's Minimum Essential Medium containing Hank's Balanced Salt Solution supplemented with 0.006 ug/ml insulin, 10 nM hydrocortisone, 0.01 M HEPES buffer, 50 ug/ml gentamicin, 100 U/ml penicillin, 100 ug/ml streptomycin, and 5% charcoal-stripped fetal calf serum at 37°C in a 5% CO_2 atmosphere. We found that these cells grew very slowly in the culture conditions needed for our experiments and thus, to allow us to proceed more quickly with these experiments, we utilized the more rapidly growing (at least under our cell culture conditions) T47D human breast cancer cell line. The proliferation of T47D cells have also been reported to be inhibited by calcitriol.

We investigated several methods to rapidly and easily assess cell number for these experiments. Actual cell counts using a hemocytometer were found to be highly variable due to the propensity of the T47D cells to aggregate. However, DNA content and protein content yielded equivalent information so for these first experiments, the protein content/cell culture well was used to assess cell number. Using a large number of measurements and with scrupulous attention to the dispersal of cell suspensions, we were able to derive the following conversion: 0.134 ± 0.068 mcg DNA/ 10^3 growing T47D human breast cancer cells (N=34).

The T47D cells were grown for 7 days in phenol red free media and charcoal treated fetal bovine serum in the presence or absence of each of the following hormonal treatments: estradiol alone (10^{-6} to 10^{-9} M), tamoxifen (10^{-7} M) alone or tamoxifen (10^{-7} to 10^{-9} M) with 10^{-8} M estradiol, retinoic acid (10^{-6} to 10^{-10} M), calcitriol (10^{-8} M), cortisone (10^{-7} to 10^{-9} M), triiodothyronine (10^{-7} to 10^{-9} M), progesterone (10^{-6} to 10^{-10} M), testosterone(10^{-6} to 10^{-10} M), or dexamethasone (10^{-7} to 10^{-9} M) (Project Objective 1). Each hormonal treatment was performed in triplicate. The experiments with estradiol and calcitriol were repeated at least twice. No consistent dose-related effect on the growth of this human breast cancer cell line as assessed by cell number was observed. Representative samples of our data are given in figures 1, 2, and 3. None of the treatments illustrated in these figures were significantly different from control cultures.

Because of the lack of an effect of calcitriol on T47D cell growth over a 7 day treatment period, we postulated that our T47D cells had lost, over many passages, the ability to respond to vitamin D. Thus, we decided to screen a large number of human breast cancer cell lines for an effect of calcitriol on breast cancer cell proliferation. These experiments were done in conjunction with Dr. William Lasswell in our laboratory. The following human cell lines were purchased from the ATCC and screened in triplicate in phenol red free media and charcoal treated fetal bovine serum with or without calcitriol (10^{-8} M): T47D (a new culture), CRL-1500, CRL-1897, HTB-20, HTB-23, HTB-24, HTB-

26, HTB-27, HTB-30, HTB-122, HTB-126, HTB-131. The results of these experiments are given in Table 1. Again, no consistent significant effect of calcitriol on cell growth was observed. The presence of the vitamin D receptor was verified in several cell lines (T47D, HTB-20, CRL1500) by the polymerase chain reaction.

We hypothesized that since the T47D cell line did contain the vitamin D receptor and had the potential to respond to calcitriol, that higher concentrations of this vitamin D metabolite would be required to suppress cell proliferation. However, higher concentrations of calcitriol would cause hypercalcemia if used in vivo and thus, its use would have limited clinical utility as a possible therapeutic agent in treating breast cancer. Several potent calcitriol analogues have recently been developed which do not have the hypercalcemic effects of the parent compound. Thus, in conjunction with Dr Daniel Bikle (while temporarily assigned to Walter Reed Army Medical Center from the University of California, San Francisco), T47D cells were exposed to calcitriol (table 2) and three calcitriol analogues: 22-oxacalcitriol (table 3), 16ene-Calcitriol (Table 4), and 16ene, 23 yne-Calcitriol (Table 5) in the presence or absence of estradiol to determine if there was any synergy/ antagonism between these hormones on cell growth (Project Objective 2). Cellular proliferation was assessed in these experiments by ^{3}H-thymidine incorporation and all treatments were done in triplicate or quadruplicate. These experiments were repeated using three other human breast cancer cell lines: MCF-7, HTB-20, and HTB-131 (data not given). In summary, estradiol had no

effect in HTB-20 and HTB-131 cells and the greatest enhancing effect on the cellular proliferation of MCF-7 cells. In none of the cell lines tested was there an antiproliferative effect of calcitriol or three of its analogues over a 6 log concentration range. Estradiol was not observed to have any synergistic effects with calcitriol.

CONCLUSIONS

Calcitriol does not appear to be a modulator of cell growth in the cultured human breast cancer cell lines tested. While some breast cancers cells may respond to hormonal manipulation (e.g. MCF-7 cells to estradiol treatment but not HTB-20 or HTB-131 cells as noted above), use of calcitriol or its analogues does not appear to be a promising therapeutic approach to control the proliferation of breast cancer cells. However, immortalized cell culture cells may represent a poor model for studying the hormonal effects of calcitriol on cellular proliferation perhaps through the loss over many passages of the normal vitamin D antiproliferative mechanisms. It is possible that although the vitamin D receptor was present in the breast cancer cell lines tested, the concentration of this receptor may not be sufficient for calcitriol to have an antiproliferative effect in these cells. Also, other cofactors lacking in our culture conditions or intracellular factors necessary for vitamin D receptor action such as the RXR receptor (Carlberg et al., 1993, Nature 361:657) may be deficient or absent in these cells. Thus, primary cultures of human breast cancers may

be a more appropriate model to screen for the effects of hormonal manipulations on breast cancer cell growth.

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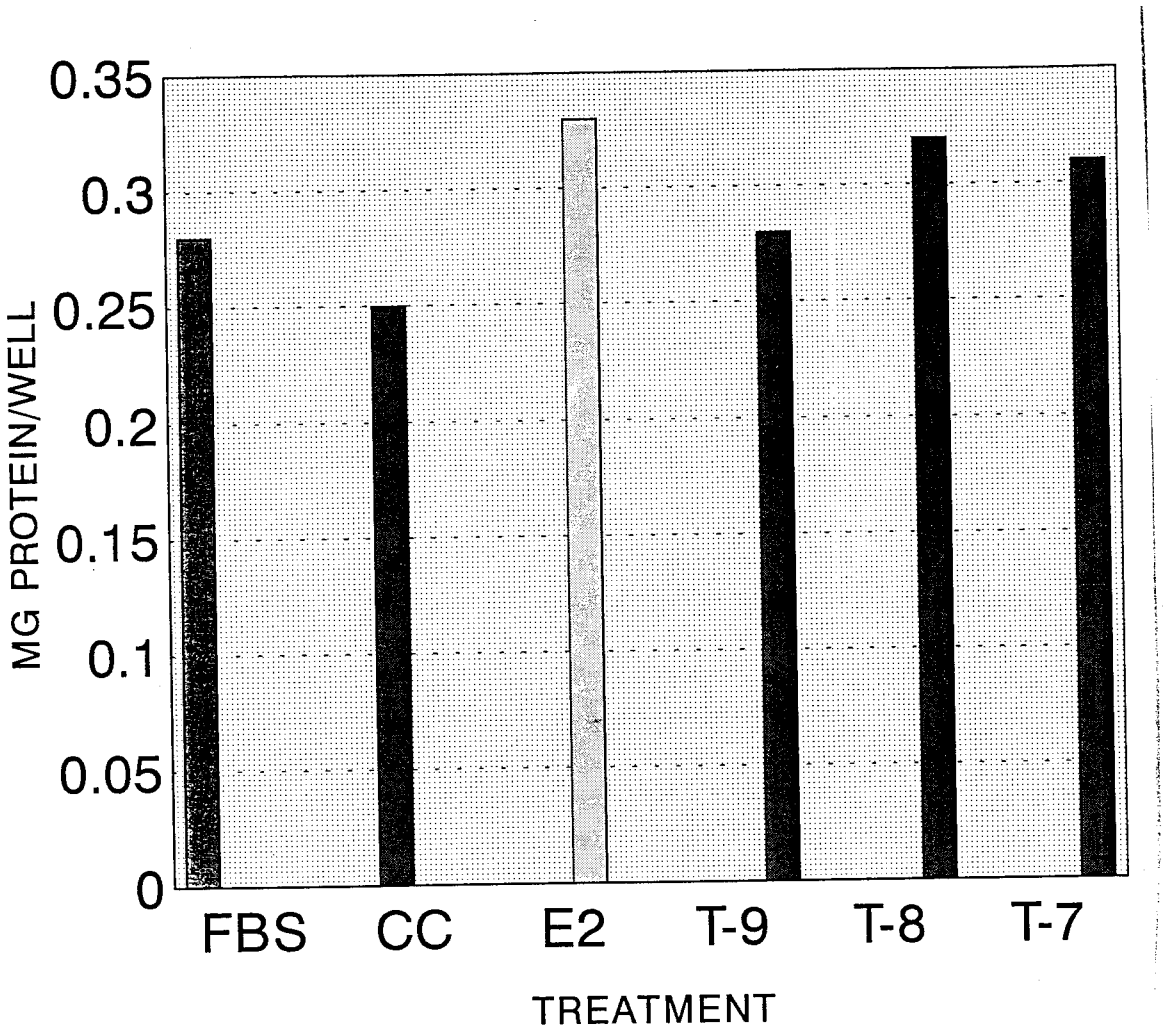
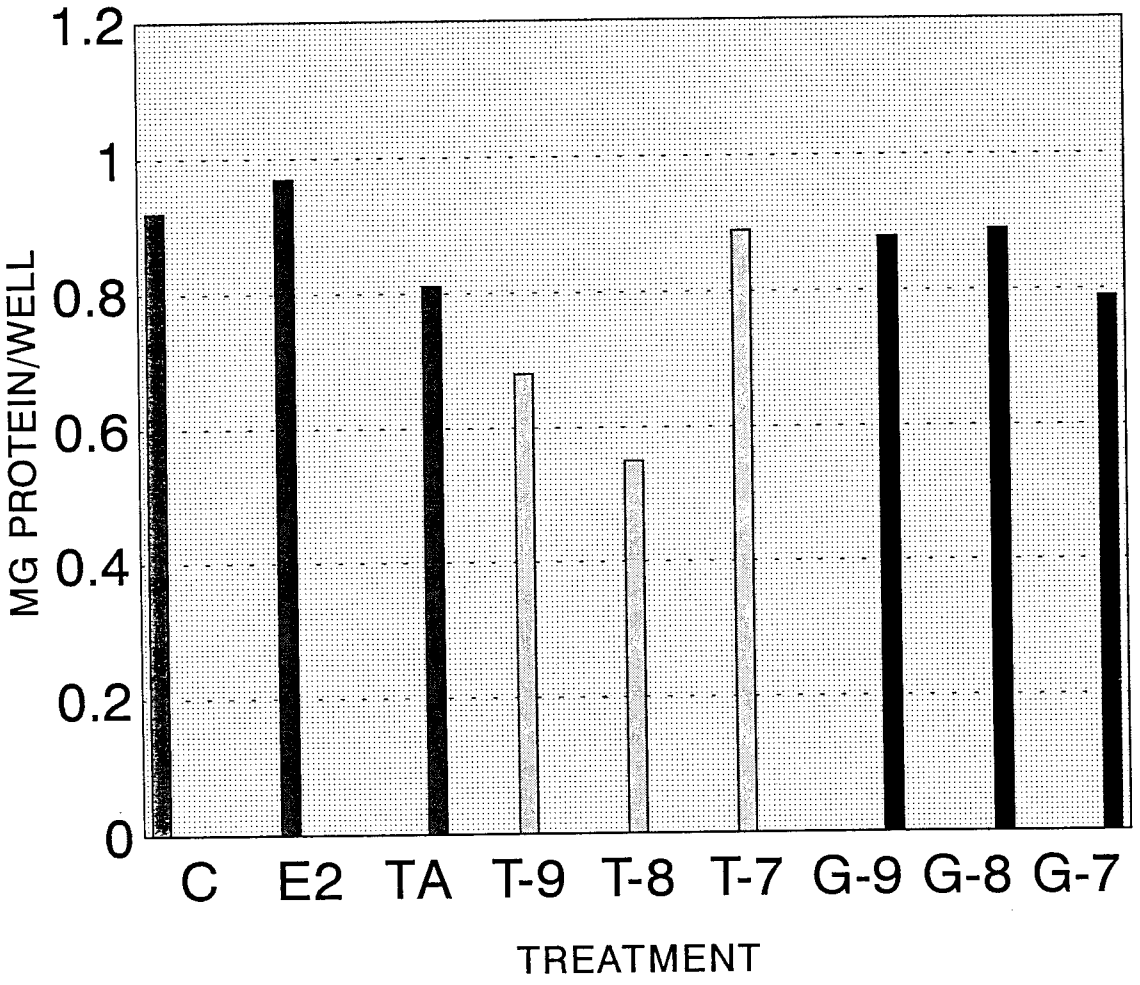
FIGURE 1: THE EFFECT OF ESTRADIOL AND TAMOXIFEN ON THE GROWTH OF T47D BREAST CANCER CELLS



The data is given as the mean of three experiments and represents the mg protein/ culture well of cells grown in the presence or absence of each hormonal treatment. The number following the letter T (eg. -9) is the log of the concentration for the hormonal treatment.

FBS= Control experiment using fetal bovine serum
CC= Control experiment using charcoal treated fetal bovine serum
E2= Estradiol (10^{-8} M)
T= Estradiol (10^{-8} M) plus Tamoxifen at the indicated concentrations

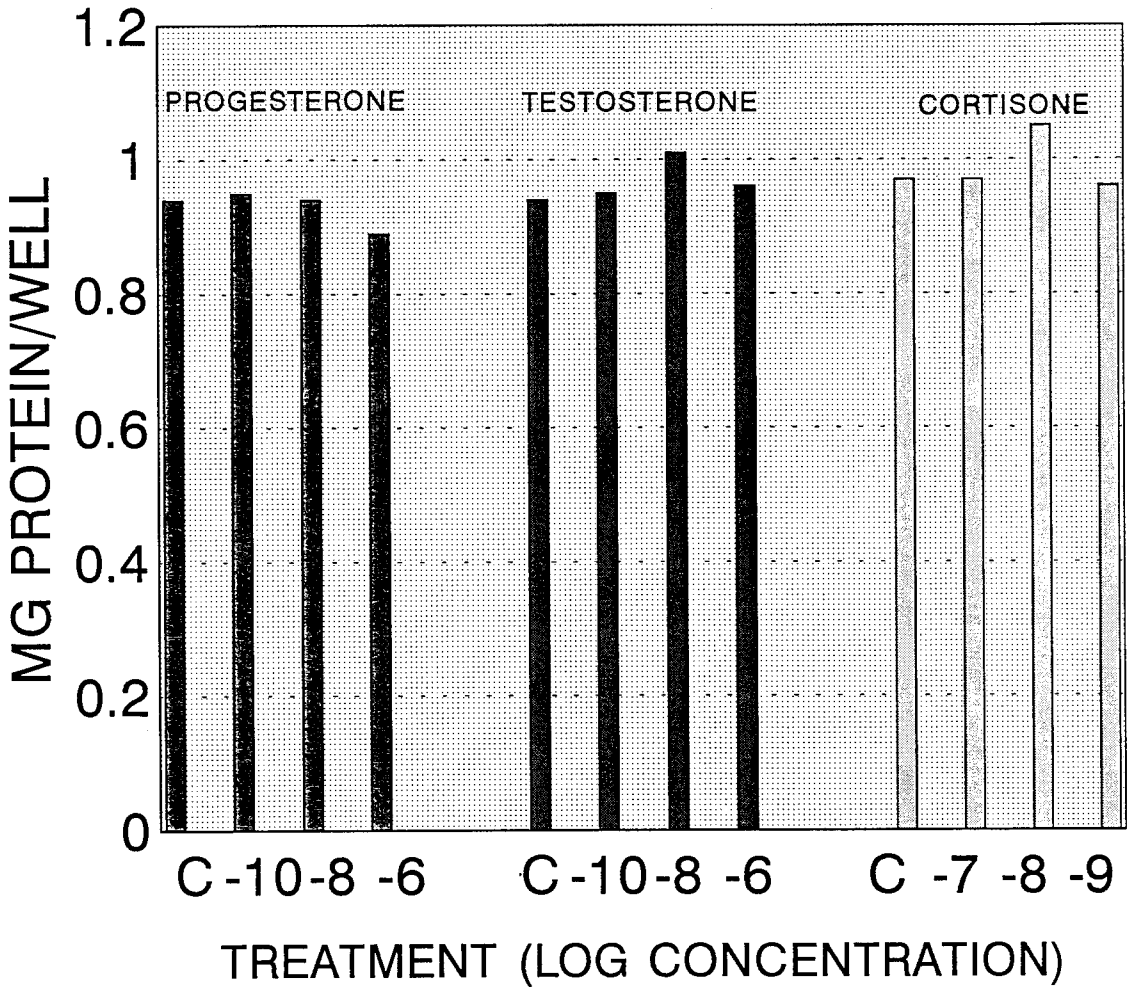
FIGURE 2: THE EFFECT OF ESTRADIOL, TAMOXIFEN, AND TRIIODOTHYRONINE ON THE GROWTH OF T47D BREAST CANCER CELLS



The data is given as the mean of three experiments and represents the mg protein/ culture well of cells grown in the presence or absence of each hormonal treatment. The number following the letter T or G (eg. -9) is the log of the concentration for the hormonal treatment.

C= Control (charcoal treated fetal bovine serum)
E2= Estradiol (10^{-8} M)
TA= Tamoxifen (10^{-7} M)
T= Estradiol (10^{-8} M) plus Tamoxifen at the indicated concentrations
G= Triiodothyronine at the indicated concentrations

FIGURE 3: THE EFFECT OF PROGESTERONE, TESTOSTERONE, AND CORTISONE ON THE GROWTH OF T47D BREAST CANCER CELLS



The data is given as the mean of three experiments and represents the mg protein/ culture well of cells grown in the presence or absence of each hormonal treatment. The number below the X axis (eg. -10) is the log of the concentration for the three hormonal treatments.

C= Control (charcoal treated fetal bovine serum)

TABLE 1: EFFECT OF CALCITRIOL (10^{-8} M) ON THE GROWTH OF HUMAN BREAST CANCER CELL LINES

RATIO: CALCITRIOL TREATED/ CONTROL[+]

CELL LINE	EXPERIMENT #1	EXPERIMENT #2	EXPERIMENT #3	EXPERIMENT #4
HTB-30	0.30	0.83	0.96	
T47D	1.06	1.03	0.94	
CRL-1500	0.84*	0.93	1.07	
HTB-26	0.82*	1.03	0.98	1.09*
HTB-27	1.00	1.29		
HTB-20	0.63*	0.90	2.21	0.70*
HTB-122	1.31*	0.98	1.00	0.95
HTB-24	0.85*	0.82		
HTB-23	0.81	0.91		
HTB-131	1.04	1.07	0.45*	
CRL-1897	0.93	0.87	1.00	
HTB-126	1.09			

[+] A ratio < 1.0 indicates inhibition of cell growth by calcitriol; a ratio > 1.0 indicates stimulation of cell growth.
* difference from a ratio = 1.0 (no effect), $p<0.05$.

TABLE 2: EFFECT OF CALCTRIOL AND ESTRADIOL ON ^{3}H-THYMIDINE INCORPORATION INTO T47D HUMAN BREAST CANCER CELLS

CALCITIOL CONCENTRATION	^{3}H-Thymidine uptake (DPM x 10^{-3}/well) CONTROL	ESTRADIOL (10^{-10} M)	p
ETHANOL	1.4+0.2	4.0+0.6	0.003
4 x 10^{-12} M	1.5+0.2	4.9+2.3	ns
4 x 10^{-11} M	1.3+0.4	5.7+2.2	0.03
4 x 10^{-10} M	2.2+0.5	7.2+2.5	0.02
4 x 10^{-9} M	1.6+0.1	5.1+2.6	ns
4 x 10^{-8} M	2.4+0.8	8.9+0.6	<0.0001
4 x 10^{-7} M	2.7+0.9*	7.3+1.1	0.0009
4 x 10^{-6} M	1.9+0.9	4.7+0.6	0.09

Data given as the mean + 1SD (N=3 or 4)

* different from other control group means (P<0.05, Tukey method)

TABLE 3: EFFECT OF 22-OXACALCITRIOL (OCT) AND ESTRADIOL ON ^{3}H-THYMIDINE INCORPORATION INTO T47D HUMAN BREAST CANCER CELLS

^{3}H-Thymidine uptake
(DPM x 10^{-3}/well)

OCT CONCENTRATION	CONTROL	ESTRADIOL (10^{-10} M)	p
CONTROL	1.4+0.2	4.0+0.7	0.0025
4 x 10^{-12} M	2.8+0.6*	5.6+0.4	0.002
4 x 10^{-11} M	2.0+0.9	6.4+1.1**	0.0009
4 x 10^{-10} M	1.7+0.01	5.2+1.1	0.008
4 x 10^{-9} M	1.9+0.2	5.4+1.8	0.03
4 x 10^{-8} M	1.4+0.3	5.3+0.7	0.001
4 x 10^{-7} M	1.7+0.2	4.3+1.2	0.02
4 x 10^{-6} M	1.2+0.1	3.2+0.4	0.01

Data given as the mean + 1SD (N=3 or 4)

* different from other control group means ($P<0.05$, Tukey method)

** different from other estradiol group means ($P<0.05$, Tukey method)

TABLE 4: EFFECT OF 16ene-CALCITRIOL (16ene-CT) AND ESTRADIOL ON ^{3}H-THYMIDINE INCORPORATION INTO T47D HUMAN BREAST CANCER CELLS

16ene-CT CONCENTRATION	^{3}H-Thymidine uptake (DPM x 10^{-3}/well) CONTROL*	ESTRADIOL* (10^{-10} M)	p
CONTROL	4.5+1.0	1.7+0.1	0.01
4 x 10^{-12} M	4.6+1.1	2.8+0.9	0.05
4 x 10^{-11} M	4.3+1.1	2.9+0.9	ns
4 x 10^{-10} M	4.5+1.6	2.7+0.7	ns
4 x 10^{-9} M	7.1+2.1	2.2+0.3	0.05
4 x 10^{-8} M	4.7+0.3	3.5+0.9	ns
4 x 10^{-7} M	6.2+1.3	2.8+1.2	0.008
4 x 10^{-6} M	5.2+1.8	3.5+0.7	ns

Data given as the mean + 1SD (N=3 or 4)

* No difference among control and estradiol groups (ANOVA)

TABLE 5: EFFECT OF 16ene,23yne-CALCITRIOL (23yne-CT) AND ESTRADIOL ON ^{3}H-THYMIDINE INCORPORATION INTO T47D HUMAN BREAST CANCER CELLS

	^{3}H-Thymidine uptake (DPM x 10^{-3}/well)		
23yne-CT CONCENTRATION	CONTROL*	ESTRADIOL (10^{-10} M)	p
CONTROL	4.5+1.0	1.7+0.1	0.01
4 x 10^{-12} M	4.2+1.8	2.7+0.4	ns
4 x 10^{-11} M	6.1+2.5	3.1+0.5**	ns
4 x 10^{-10} M	4.1+1.1	3.0+1.0**	ns
4 x 10^{-9} M	3.2+0.3	3.4+0.8**	ns
4 x 10^{-8} M	5.0+1.1	3.5+0.2**	ns
4 x 10^{-7} M	4.9+1.9	1.8+0.2	0.05
4 x 10^{-6} M	3.4+0.6	2.3+0.3	0.02

Data given as the mean + 1SD (N=3 or 4)

* no differences from the control group mean

** difference from the estradiol treated control group ($p<0.05$, Tukey method)