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Inhibition of Human Breast Cancer Cell Proliferation in Tissue Culture by the Neuroleptic Agents Pimozide and Thioridazine[1]

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ABSTRACT

Permanent cell culture lines derived from human breast cancer tissue are important experimental models in the study of human breast cancer cell proliferation. In the present work, pimozide, thioridazine, W-13, and W-12 were shown to inhibit MCF-7 human breast cancer cell growth. The 50% inhibition concentration values determined in two proliferation assays, [^{3}H]thymidine incorporation and cell number, were in close agreement for each compound tested. The order of potency for growth inhibition in the presence of 2% stripped calf serum was pimozide (K_i 2 μM) > thioridazine (K_i 5 μM) > W-13 (K_i 15 μM) > W-12 (K_i 39 μM). Similar concentrations of these compounds blocked estradiol-induced growth of MCF-7 cells, but estrogen receptor (ER) interactions do not seem to be involved. Pimozide and thioridazine had no effect on the estradiol binding properties of the MCF-7 ER, nor did pimozide interfere with the induction of progesterone receptors by estradiol. Furthermore, pimozide also inhibited incorporation of [^{3}H]thymidine into MCF-7 cells stimulated by polypeptide hormones in serum-free medium. The K_i for pimozide in serum-free medium alone, 0.46 μM, was similar to that determined in the presence of insulin (0.42 μM), insulin-like growth factor I (0.54 μM), and epidermal growth factor (0.43 μM). The effects of pimozide on breast cancer cell growth were not limited to the MCF-7 cell line. Pimozide also blocked cell growth and [^{3}H]thymidine incorporation into the ER-positive T47D and ZR75-1B human breast cancer cell lines and the ER-negative human breast cancer cell line, MDA-MB-231. Although numerous mechanisms of action of pimozide and thioridazine have been identified, both drugs are calmodulin antagonists at drug concentrations that inhibit breast cancer cell growth *in vitro*. Inhibition of MCF-7 cell growth by the selective calmodulin antagonists W-13 and W-12 is consistent with a role for calmodulin antagonism in the broad growth-inhibitory properties of pimozide. We conclude that pimozide and thioridazine may be useful in the control of estradiol- and polypeptide hormone-induced growth of ER-positive and ER-negative human breast tumors.

INTRODUCTION

Estrogens are involved in the etiology and growth of human breast cancer and are thought to exert their effects via binding to specific, high affinity estrogen receptors. The presence of ER[3] in human breast cancer is predictive of hormone-dependent tumors which regress in response to endocrine therapies in about 50% of patients (1). The remaining ER-positive and ER-negative tumors are generally resistant to endocrine therapies, and these are classified as "hormone independent." Alternative therapies for estrogen-dependent tumors and for tumors that are resistant to existing endocrine treatments may be possible based upon an understanding of the mechanisms controlling breast cancer cell proliferation in response to polypeptide hormones. Insulin, insulin-like growth factor I and II, epidermal growth factor, transforming growth factor-α, and basic fibroblast growth factor have been shown to stimulate proliferation of human breast cancer cells *in vitro* (2). Autocrine and paracrine production of these or related polypeptide growth factors may explain the estrogen-independent growth of certain ER-positive and ER-negative human breast cancers (3).

Recent studies of the mechanism of growth regulation by polypeptide hormones in fibroblasts indicate that several intracellular signal transduction pathways are utilized in the transmission of the mitogenic stimulus to the nucleus (4). Various calmodulin antagonists (5–10) and protein kinase C inhibitors (11–14) have been shown to inhibit cell growth *in vitro*, but pharmacological agents with multiple intracellular actions may be particularly effective inhibitors of cell proliferation. Tamoxifen, an antiestrogen used widely in the treatment of ER-positive human breast cancers (15), exerts multiple pharmacological effects in addition to inhibition of estrogen-receptor interactions. These include binding to a distinct antiestrogen binding site (16), receptors for histamine (17) and dopamine (18), inhibition of calmodulin (19) and protein kinase C activation (20), and blockade of voltage-dependent plasma membrane calcium channels (21). High concentrations of tamoxifen inhibit growth of ER-negative human breast cancer cell lines in tissue culture (22). These ER-independent mechanisms may contribute to breast cancer growth inhibition by tamoxifen.

In the experiments described in this report, pimozide inhibited the proliferation of ER-positive and ER-negative human breast cancer cell lines grown in 2% charcoal-stripped calf serum *in vitro*. In MCF-7 cells, pimozide also blocked estrogen-induced growth independently of effects on the ER and proliferation induced by the polypeptide hormones insulin, IGF-I, and EGF. These results in tissue culture were observed with therapeutic drug concentrations, suggesting that pimozide and thioridazine may be of practical value in the management of human breast cancer *in vivo*.

MATERIALS AND METHODS

Cell Culture. Three ER-positive human breast cancer cell lines were used in these studies, MCF-7 (passage 24–69) (23), ZR75-1B (24), T47D (25), and one ER-negative human breast cancer cell line, MDA-MB-231 (26). Cells were grown in DMEM plus 10% FCS (Hazleton Research Products, Inc., Lenexa, KS) and 2 mM glutamine at 37°C in a humidified 5% CO_2-95% air incubator. Experiments were conducted in phenol red-free DMEM (Hazleton Research Products) and 2% stripped CS (GIBCO Laboratories, Grand Island, NY), treated to remove endogenous steroids. To prepare stripped CS, a 0.25% dextran-2.5% Norit A (dextran-coated charcoal) suspension in 0.01 M Tris, pH 7.9, was centrifuged (1,500 × *g*, 15 min) to pellet the charcoal. One hundred ml of CS were incubated for 30 min at 56°C with the pellet

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[3] The abbreviations used are: ER, estrogen receptor(s); CS, calf serum; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; estradiol, 1,3,5-(10)-estratriene-3,17/β-diol; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution, 0.4 g/liter KCl-0.06 g/liter KH_2PO_4-8 g/liter $NaHCO_3$-0.09 g/liter Na_2HPO_4 $7H_2O$; IGF-I, insulin-like growth factor I; pimozide, 1-(1-(4,4-bis(4-fluorophenyl)-butyl)-4-piperidinyl)-1,3-dihydro-2H-benzimidazol-2-one; TED, 0.01 M Tris[hydroxymethyl]aminomethane-1.5 mM disodium EDTA-5 mM dithiothreitol, pH 7.4; thioridazine, 10-[2-(1-methyl-2-piperidyl)ethyl]-2-(methylthio)phenothiazine; W-12, *N*-(4-aminobutyl)-2-naphthalenesulfonamide hydrochloride; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide hydrochloride.

from 20 ml of 0.25% dextran-2.5% Norit A suspension. The CS was centrifuged (1,500 × *g*, 15 min) to remove the charcoal and then incubated for 2 h at 37°C with 2 units/ml of type IV sulfatase from limpets (Sigma, St. Louis, MO). The initial treatment with dextran-Norit A was repeated, followed by a 30-min centrifugation at 12,000 × *g*. The stripped serum was sterilized by filtration through a 0.22-μm pore filter and stored frozen at −20°C until use.

Drugs and Hormones. Pimozide (Lemmon Co., Sellersville, PA) was prepared in dimethyl sulfoxide and stored at −20°C. Thioridazine (Smith, Kline, and Beckman, Philadelphia, PA), W-13, and W-12 (Seikagaku America, Inc., Rockville, MD) were prepared fresh in water for each experiment and sterilized by filtration.

Estradiol-17β (Steraloids, Pawling, NY) and [2,4,6,7-^{3}H]estradiol (91 Ci/mmol; Amersham Corp., Arlington Heights, IL) were stored in 95% ethanol at −20°C. Promegestone (R5020) and [17α-methyl-^{3}H]promegestone (82 Ci/mmol) were purchased from DuPont (Wilmington, DE) and stored in 95% ethanol in the dark at −20°C. Insulin (bovine pancreatic), insulin-like growth factor I (human, recombinant), and epidermal growth factor (mouse submandibular gland) were purchased from Boehringer-Mannheim (Indianapolis, IN) and stored at 4°C as sterile saline solutions.

Cell Growth Determinations. Cells were replica plated (0.5–3 × 10^5 cells) in T-25 flasks in DMEM plus 10% FCS and 2 mM glutamine. The plating medium was replaced three times at 6–8-h intervals with fresh 3-ml aliquots of phenol red-free DMEM plus 2% stripped CS, 2 mM glutamine, and 50 μg/ml gentamicin. Drug, estradiol, or the appropriate solvent controls were added to the flasks after a total of 24 h in steroid-free medium. On the days indicated, cells in the medium and those harvested from the monolayer by trypsinization were pooled, pelleted by centrifugation at 1000 × *g* for 5 min, and resuspended in 1 ml of HBSS. Single cell suspensions were made by passing the cells through a 20-gauge needle. Cell counts were performed using a hemacytometer in the presence of 0.06% trypan blue to assess viability.

[^{3}H]Thymidine Incorporation Assay. Cells were replica plated (1–2 × 10^5/well) in 6-well dishes on day 1 in phenol red-free DMEM plus 2% stripped CS. The medium was replaced with fresh phenol red-free DMEM plus 2% stripped CS twice on day 3 and once on day 4. Drugs and/or hormones were added to the cells following the final medium replacement on day 4. Eighteen to 24 h later, cells were incubated at 37°C for 1 h with 0.5 μCi/well of [^{3}H-methyl]thymidine (85 Ci/mmol; DuPont). Cells were then placed on ice, washed three times with HBSS, and harvested using HBSS plus 0.1% EDTA. Cell pellets were sonicated, and aliquots were assayed for [^{3}H]thymidine incorporation into trichloroacetic acid precipitates, as detailed previously (27).

Flow Cytometry. Cells were replica plated (4 × 10^5/60-mm^2 dish) on day 1 in 3 ml of phenol red-free DMEM plus 2% stripped CS. Medium replacements and drug/hormone additions were performed according to the schedule used in the [^{3}H]thymidine incorporation assays. Cells were harvested and counted after 24 or 72 h of drug and/or hormone exposure. Nuclei were prepared and stained with propidium iodide according to the method of Vindeløv (28), except that nuclei were treated with trypsin (0.03 mg/ml) for 25 min at 37°C before proceeding with the ribonuclease and propidium iodide staining steps. The propidium iodide-stained nuclei from 1 × 10^6 cells were analyzed using a FACScan Instrument (Becton-Dickinson, Mountainview, CA) equipped with the Consort30 DNA software package. The distribution of cells in G_1/G_0, S, and G_2-M phases of the cell cycle was determined by the sum of broadened rectangles method.

Estrogen Receptor Measurements. MCF-7 cells were replica plated (1–4 × 10^5/well) in 6-well dishes in DMEM plus 10% FCS. Twenty-four h later, the plating medium was replaced with phenol red-free DMEM plus 2% stripped CS for at least 24 h. Immediately before the addition of [^{3}H]estradiol, 2 ml/well of serum-free, phenol red-free DMEM were added. Whole cell [^{3}H]estradiol binding assays were performed as detailed previously using sodium hydroxide to solubilize the cells (29). The number and affinity (K_d) of the ER sites were determined by computer Scatchard plot analysis (30). Competition between unlabeled estradiol, pimozide, thioridazine, and [^{3}H]estradiol for receptor binding was determined in an identical manner using [^{3}H]estradiol at a concentration of 5 × 10^{-10} M and the indicated concentrations of unlabeled competitors.

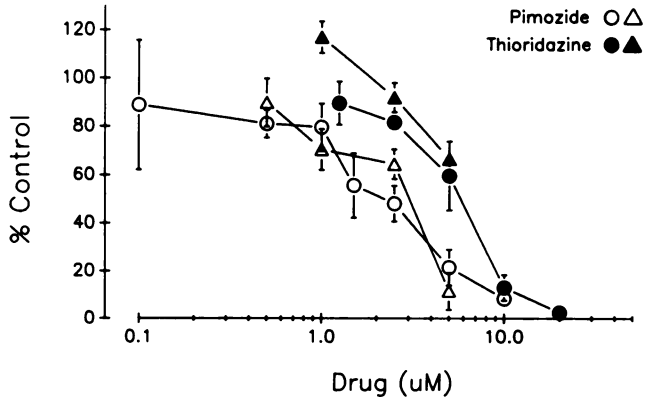

Fig. 1. Inhibition of MCF-7 proliferation by pimozide and thioridazine. Proliferation was measured as [^{3}H]thymidine incorporation during a 1-h pulse in cells incubated for 24 h with pimozide (△) or thioridazine (▲), or as the number of viable cells after a 5-day incubation with pimozide (○) or thioridazine (●). Data are expressed as the mean percentage of solvent controls ± SE (*bars*) of 2–5 experiments.

Nuclear estrogen receptors were measured using an exchange assay and a single saturating concentration of [^{3}H]estradiol (31). Crude nuclei (see below) were resuspended in 1.5 ml T buffer, and aliquots were incubated with 10 nM [^{3}H]estradiol in the presence and absence of 1 μM unlabeled diethylstilbestrol. Exchange reactions were conducted at 30°C for 30 min followed by a 30-min incubation at 37°C. Reactions were terminated by dilution and three washes with 1.5 ml of ice-cold TED buffer. Nuclear bound [^{3}H]estradiol was quantitated by scintillation counting ethanol extracts of the washed nuclear pellets and corrected for the amount of protein. In all estrogen receptor determinations, competed and uncompeted samples were assayed in triplicate.

Progesterone Receptor Measurements. MCF-7 cells were plated at low density (2–5 × 10^5 cells/T-75 flask), grown for 10 days in phenol red-free DMEM plus 2% stripped CS, and then exposed to estradiol, pimozide, or the combination of pimozide and estradiol for 72 h. Cells were harvested by trypsinization, washed in HBSS, resuspended in 1 ml of ice-cold TED, and lysed using 50 strokes of a tight-fitting pestle in a Dounce homogenizer. Cytosol was prepared by sequential centrifugation at 800 × *g* for 20 min to pellet a crude nuclear fraction and at 100,000 × *g* for 40 min. Progesterone receptors in the resulting cytosol were measured using a competitive binding assay (32). A single saturating concentration of [^{3}H]R5020 (20 nM) in the absence and presence of a 100-fold excess of unlabeled R5020 was used. Both competed and uncompeted tubes contained a final concentration of 100 nM hydrocortisone (Sigma) to prevent R5020 binding to glucocorticoid receptors. Samples were incubated in triplicate at 0–4°C for 18 h. Unbound [^{3}H]R5020 was removed by a 10-min incubation (0–4°C) with dextran-coated charcoal and centrifugation (1,000 × *g*, 5 min). Aliquots of the supernatant were assayed for radioactivity and protein content as detailed previously.

RESULTS

Inhibition of Cell Growth by Pimozide and Thioridazine. The inhibition of MCF-7 cell numbers and [^{3}H]thymidine incorporation by increasing concentrations of pimozide and thioridazine is shown in Fig. 1. Qualitatively identical inhibition curves were observed when MCF-7 cells were treated with pimozide or thioridazine in the presence of 2.5 nM estradiol. Under the culture condition used, estradiol stimulated a 1.7-fold increase in cell numbers and a 4.7-fold increase in [^{3}H]thymidine incorporation compared with control cells maintained in 2% CS alone. Inhibition constants for pimozide and thioridazine were estimated from these data using the equation

$$K_i = (1/f_b - 1)\ (\text{Drug conc.})$$

where f_b = fractional drug response (Table 1). There is close agreement between the K_i determined by [^{3}H]thymidine incorporation assays following acute drug exposure (18–24 h) and cell number data obtained following a 5-day drug exposure. Under all conditions tested, pimozide was twice as potent as thioridazine in the inhibition of MCF-7 cell proliferation *in vitro*. In preliminary studies (data not shown), the potencies of trifluoperazine and fluphenazine were found to be equivalent to that of thioridazine in the inhibition of MCF-7 cell growth.

Pimozide inhibition of estradiol-induced [^{3}H]thymidine incorporation into MCF-7 cells was not overcome by increasing the concentration of estradiol to 250 nM. Pimozide (2.5 μM) inhibited [^{3}H]thymidine incorporation by 56% in cells exposed to 2.5 nM estradiol and by 61% in cells exposed to 250 nM estradiol. Therefore, competition with estradiol for ER binding sites or an increased rate of estradiol metabolism to less active metabolites is unlikely to account for the growth-inhibitory actions of pimozide. Analogous studies with thioridazine were not performed; however, the interactions of both pimozide and thioridazine with ER were further explored.

Effects of Pimozide and Thioridazine on the MCF-7 Estrogen and Progesterone Receptors. Pimozide and thioridazine failed to compete with [^{3}H]estradiol for MCF-7 ER binding sites at drug concentrations that antagonized the growth of MCF-7 cells (Fig. 2). Competition between unlabeled estradiol and [^{3}H]estradiol for ER binding is shown as a control. Changes in the ability of the estrogen receptor to bind estradiol following long-term exposure to pimozide and thioridazine were also examined. Whole cell ER binding assays were performed after exposure of MCF-7 cells to pimozide (1 μM) or the appropriate solvent (0.1% dimethyl sulfoxide) for 4 h, 24 h, or 5 days. Scatchard plot analyses of the binding data were used to estimate the number of estrogen receptor sites per cell and their affinity for estradiol. The binding data are summarized in Table 2 and indicate that neither estrogen receptor number nor affinity for estradiol was reduced by chronic exposure of MCF-7 cells to pimozide and thioridazine.

Finally, the biological activity of the estrogen receptor in pimozide-treated MCF-7 cells was demonstrated by its nuclear localization and its ability to induce cytosolic progesterone receptors (Table 3). Estrogen receptors in estrogen-deprived and estradiol-treated cells were localized primarily within the nucleus. Levels of cytosolic estrogen receptors in these same cells were less than 10% of the nuclear levels (data not shown). Pimozide treatment did not reduce levels of nuclear ER or increase levels of cytosolic ER.

PgR were undetectable in MCF-7 cells grown in the complete absence of estrogen for 10 days. Induction of PgR by treatment with 2.5 nM estradiol for 72 h occurred in both the absence and the presence of 2.5 μM pimozide. These data suggest that pimozide interferes with the process of estrogen-induced growth in MCF-7 cells via a mechanism that is distinct from that involved in the induction of progesterone receptors.

Effects of Pimozide on MCF-7 Cell Cycle. To determine the influence of pimozide on progression of MCF-7 cells through the cell cycle, MCF-7 cells were treated for 24 or 72 h with estradiol, pimozide, or estradiol plus pimozide (Fig. 3). Pimozide treatment resulted in a concentration- and time-dependent decrease in the percentage of cells in S phase. Prolonged exposure to pimozide resulted in a gradual accumulation of cells in G_1/G_0. This effect was concentration dependent and most marked in the estradiol-treated cells that were traversing the cell cycle most rapidly. MCF-7 cells exposed to high concentrations of pimozide (10 μM) in the absence of estradiol showed some accumulation in G_2-M, an effect that is sometimes indicative of cytotoxicity (33). Cell viability was monitored throughout these experiments by trypan blue exclusion, which corroborated this observation. At 24 h, cell viability exceeded 90% in all cells exposed to 0 or 2.5 μM pimozide and 85% in cells exposed to 5 or 10 μM pimozide. By 72 h, cell viability in untreated cells was 88%, but it decreased to approximately 65 and 48% in cells treated with 5 or 10 μM pimozide, respectively. We conclude that a delay in the G_1/G_0 to S phase transition plays a role in the inhibition of MCF-7 cell growth by concentrations of pimozide <5 μM and that higher concentrations of pimozide are cytotoxic.

Inhibition of Polypeptide Hormone-induced Growth of MCF-7 Cells by Pimozide. The inhibition of polypeptide hormone-induced MCF-7 cell proliferation was measured in [^{3}H]thymidine incorporation assays in which the final medium change contained no serum. The lowest concentrations of insulin, IGF-I, and EGF that stimulated maximal [^{3}H]thymidine incorporation under these conditions were determined and used in these studies. Parallel MCF-7 cultures were stimulated with insulin, EGF, IGF-I, and estradiol for 18 h in the presence and absence of pimozide. The K_i values for pimozide calculated for all three polypeptide hormones were indistinguishable (Table 4). Pimozide inhibited estradiol-stimulated [^{3}H]thymidine incorporation in serum-free medium with a K_i of 0.25 ± 0.12 μM. The similarity in the inhibition constants for pimozide in unstimulated cells and in cells treated with insulin, IGF-I, EGF, or

Table 1 *Inhibition constants for pimozide and thioridazine in MCF-7 cell proliferation assays*

Drug	K_i (μM) [^{3}H]Thymidine incorporation assay[a]	K_i (μM) Cell no.[b]
Pimozide		
Control	2.1 ± 0.5	3.0 ± 0.9
Estradiol (2.5 nM)	2.2 ± 0.3	1.3 ± 0.4[c]
Thioridazine		
Control	5.9 ± 2.1	ND[d]
Estradiol (2.5 nM)	4.4 ± 1.7	5.1 ± 1.8

[a] Data are the mean ± SE of 2–5 experiments performed in triplicate.
[b] Data are the mean ± SE of 3 experiments.
[c] $P < 0.05$, Student's t test.
[d] Not determined.

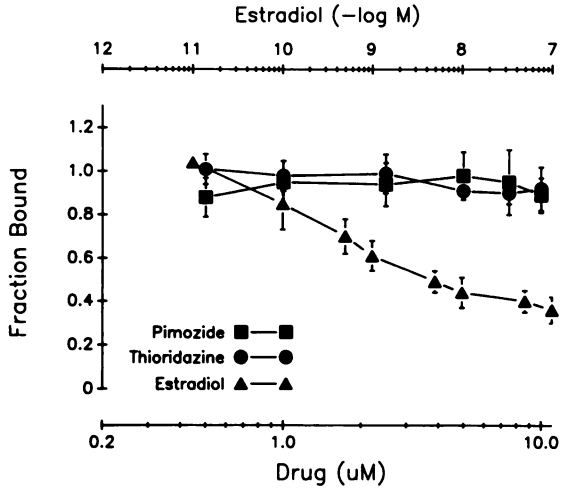

Fig. 2. Competition between the thioridazine, pimozide, and estradiol for ER binding in intact MCF-7 cells. Cells were incubated with serum-free phenol red-free DMEM containing 0.5 nM [^{3}H]estradiol alone or in the presence of the unlabeled competitors at the concentrations indicated for 40 min at 37°C. Specific [^{3}H]estradiol binding in the absence of competitor is equal to 1. Data represent the mean ± SE (*bars*) of 3 experiments performed in triplicate.

Table 2 *Estrogen receptors in MCF-7 cells after acute and chronic exposure to pimozide and thioridazine*

Time	ER (sites/cell)[a]			
	Control	Pimozide (1 μM)	Control	Thioridazine (2.5 μM)
4 h	165,424 ± 60,000[b] (K_d = 0.44 ± 0.13 pM)[c]	153,353 ± 28,029 (K_d = 0.56 ± 0.11 pM)	133,568 ± 27,699 (K_d = 0.44 ± 0.13 pM)	142,877 ± 21,760 (K_d = 0.46 ± 0.13 pM)
24 h	191,398 ± 19,767	171,976 ± 46,699 (K_d = 0.39 ± 0.12 pM)	169,965 ± 54,597	202,872 ± 65,985 (K_d = 0.44 ± 0.09 pM)
5 days	120,297 ± 4,541[b]	137,496 ± 22,480[b] (K_d = 0.33 ± 0.13 pM)	195,213 ± 80,540[b]	297,904 ± 61,792[b] (K_d = 0.37 ± 0.11 pM)

[a] Data represent the mean ± SE of 3 separate experiments or mean ± range of 2 experiments.
[b] n = 2.
[c] The K_d of control cells is the mean of 15 separate experiments that include 4-h, 24-h, and 5-day treatment groups.

Table 3 *Effect of pimozide on nuclear estrogen receptor (ER_n) and PgR levels*

	ER_n[a] (fm/mg protein)	PgR[b] (fm/mg protein)
Control	80 ± 62	0
Estradiol (2.5 nM)	106 ± 54	52 ± 4
Pimozide (2.5 μM)	90 ± 38	2.5 ± 2.5
Pimozide (2.5 μM) + estradiol (2.5 nM)	104 ± 40	44 ± 15

[a] ER_n levels are the mean ± SD of triplicate determinations in 6–7 experiments.
[b] PgR levels are the mean ± range of triplicate determinations in 2 experiments.

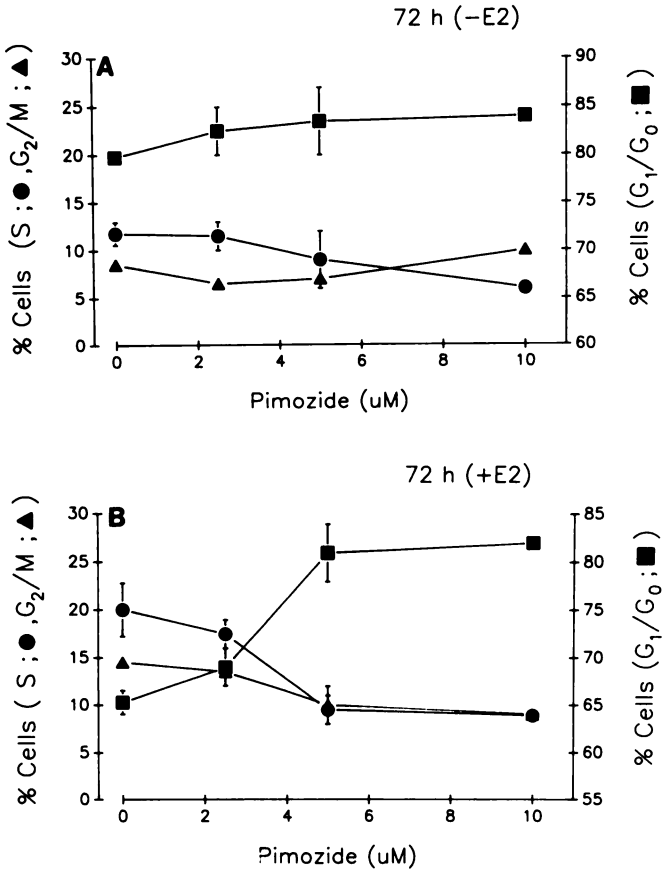

Fig. 3. Cell cycle effects of pimozide in MCF-7 cells. Cells were incubated in phenol red-free DMEM plus 2% stripped CS containing the indicated concentrations of pimozide in the absence (*A*) or presence (*B*) of 2.5 nM estradiol for 72 h. The percentage of cells in G_1/G_0 (■), S (●), and G_2-M (▲) phase were determined by flow cytometry. Data represent the mean ± SE (*bars*) of 2–3 experiments.

Table 4 *Effect of pimozide on polypeptide hormone-induced [^{3}H]thymidine incorporation into MCF-7 cells in serum-free medium*

Hormone	Maximal fold induction of [^{3}H]thymidine incorporation[a]	K_i pimozide[b] (μM)
None	1.00	0.46 ± 0.02
Insulin (3 ng/ml)	3.20 ± 0.68 (n = 3)	0.42 ± 0.01
IGF-I (0.5 ng/ml)	1.97 ± 0.14 (n = 4)	0.54 ± 0.20
EGF (1 ng/ml)	1.40 ± 0.11 (n = 2)	0.43 ± 0.09

[a] Data represent mean ± SE of 3–4 experiments or mean ± range of 2 experiments performed in triplicate.
[b] Data represent mean ± SD.

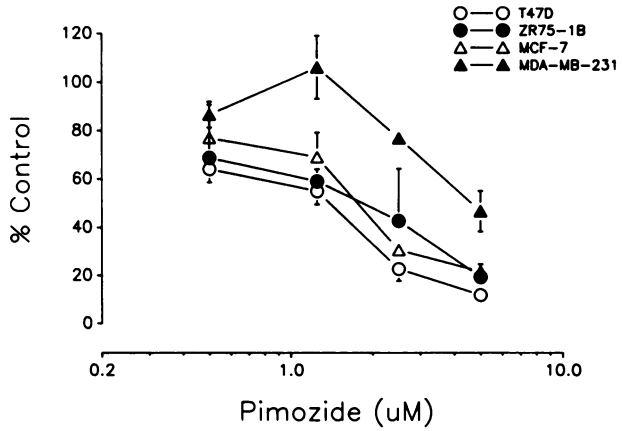

Fig. 4. Comparison of pimozide effects in ER-positive and ER-negative human breast cancer cell lines. Equal numbers of MCF-7 (△), T47D (○), ZR75-1B (●), and MDA-MB-231 (▲) cells were incubated for 18 h with pimozide or solvent alone and then pulse labeled with [^{3}H]thymidine. [^{3}H]Thymidine incorporation is expressed as the mean percentage of control cells ± SE (*bars*) of 2 (ZR75-1B) or 4 experiments performed in triplicate.

estradiol is consistent with a common mechanism of growth inhibition, but further experimentation is needed to address this hypothesis. The K_i values reported in Table 4 are lower than shown in Table 1 and reflect the use of serum-free medium. Inclusion of 2% stripped CS to serum-free medium containing insulin raised the estimated K_i for pimozide approximately 5-fold (data not shown).

Pimozide Inhibition of Proliferation in Other Human Breast Cancer Cell Lines. [^{3}H]Thymidine incorporation assays were performed, directly comparing the effects of pimozide in three ER-positive human breast cancer cell lines (MCF-7, ZR75-1B, and T47D) and one ER-negative cell line (MDA-MB-231) during growth in phenol red-free DMEM plus 2% stripped CS (Fig. 4; Table 5). The response of all three ER-positive breast cancer cell lines to pimozide was very similar. Approximately 5 times more pimozide was required to inhibit thymidine incorporation into the ER-negative cell line, MDA-MB-231, than the most sensitive ER-positive line, T47D. To determine the relative sensitivity of all four human breast cancer cell lines to growth inhibition by pimozide, cell numbers were monitored for 6 days during growth in the presence of 5 μM pimozide. These assays showed pimozide to be an equally effective inhib-

Table 5 *Comparison of pimozide inhibition of [³H]thymidine incorporation into ER-positive and ER-negative human breast cancer cell lines*

Cell line	K_i pimozide[a] (μM)
MCF-7[b]	1.72 ± 0.32
ZR75-1B[b]	1.48 ± 0.17
T47D[b]	0.95 ± 0.17
MDA-MB-231[c]	5.28 ± 1.2[d]

[a] Data represent mean ± SE of determinations in 3–5 experiments.
[b] ER-positive cell lines.
[c] ER-negative cell line.
[d] $P < 0.05$, Newman-Keuls procedure.

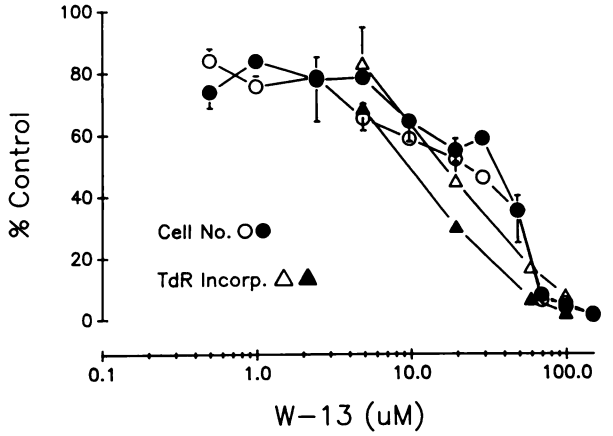

Fig. 5. Inhibition of MCF-7 cell proliferation by W-13. Proliferation was measured as [³H]thymidine incorporation during a 1-h pulse in cells incubated with W-13 for 20 h in the presence (▲) or absence (△) of 2.5 nM estradiol, or as the number of viable cells after a 5-day incubation with W-13 in the presence (●) or absence (○) of 2.5 nM estradiol. Data are expressed as the mean percentage of solvent controls ± SE (*bars*) of 2 [³H]thymidine experiments and 3–6 cell number experiments.

Table 6 *Effects of W-13 and W-12 on MCF-7 cell proliferation assays*

	K_i (μM)		
	W-13		W-12,
	[³H]Thymidine incorporation assay[a]	Cell no.[b]	[³H]thymidine incorporation assay[a]
Control	15.06 ± 3.6	11.23 ± 2.85	38.93
Estradiol (2.5 nM)	6.31 ± 2.1[c]	13.5 ± 2.96	ND[d]

[a] Data are the mean ± SE of 2 experiments performed in triplicate.
[b] Data are the mean ± SE of 3–8 experiments performed in triplicate.
[c] $P < 0.1$, Student's t test.
[d] Not determined.

itor of proliferation in both the ER-negative and ER-positive cell lines (data not shown).

Inhibition of MCF-7 Cell Proliferation by W-13 and W-12. Inhibition of MCF-7 cell numbers and thymidine incorporation by two relatively selective calmodulin antagonists, W-13 and W-12, was next examined. W-13 and W-12 differ 3.8–5.7-fold in their ability to bind calmodulin and inhibit calmodulin-dependent enzyme activation (5). Comparisons of the relative biological activity of W-13 and W-12 provide a relatively specific method for identification of calmodulin-dependent processes. The results in Fig. 5 show that there is a close correlation between the inhibition of MCF-7 cell numbers after a 5-day exposure to W-13 and inhibition of [³H]thymidine incorporation after a 20-h exposure time. Inhibition curves were essentially identical in control cells and in cells stimulated with 2.5 nM estradiol. Evidence that W-13 is acting through calmodulin is provided in Table 6, which compares the relative activity of W-13 and the dechloro derivative, W-12. The inhibition constants for W-12 (39 μM) and W-13 (6–15 μM) estimated from these data reflect the expected difference in the ability of these two compounds to inhibit calmodulin.

DISCUSSION

Calmodulin antagonism is likely to play a role in the mechanism of the antiproliferative action of pimozide and thioridazine. Our experiments with pimozide and thioridazine are consistent with earlier studies showing inhibition of the growth of MCF-7 (10, 35, 36) and other tumor cells in tissue culture (5–9) by compounds with calmodulin antagonist activity. Concentrations of pimozide and thioridazine that block breast cancer cell growth are similar to drug concentrations that have been shown to inhibit calmodulin-dependent enzyme activation (34). Furthermore, our results with the naphthalenesulfonamides W-13 and W-12 provide specific evidence for the role of calmodulin in the control of MCF-7 breast cancer cell growth *in vitro*.

Pimozide is the most potent calmodulin antagonist among the neuroleptic drugs currently approved for clinical use (34); thus, we have focused our efforts on this compound. Pimozide was shown to inhibit proliferation of one ER-negative and three ER-positive human breast cancer cell lines. In MCF-7 cells, pimozide was shown to block estradiol-induced growth independently of effects on the ER and to inhibit proliferation in response to three polypeptide hormones, insulin, IGF-I, and EGF.

A variety of compounds possessing anticalmodulin activity induce similar alterations in cell cycle kinetics. Inhibition of MCF-7 cell growth by R24571, tamoxifen, and pimozide is accompanied by a decrease in the number of cells in S phase and a concomitant increase in the number of cells in G_1/G_0 (36, 37). Sutherland and colleagues (36) have presented evidence that the actions of tamoxifen and the calmodulin antagonist R24571 are specifically directed to an event early in the G_1 phase of the MCF-7 cell cycle. These pharmacological data are consistent with a mechanism of action involving a delay in the progression of cells through G_1, and, consistent with this hypothesis, Rasmussen and Means (38) have shown that biochemically elevated intracellular calmodulin concentrations enhance the progression of mouse C127 cells through the G_1 phase of the cell cycle. The nature of the calmodulin-sensitive event(s) in G_1 is of considerable interest, as this could serve as an important target for chemotherapeutic intervention of tumor cell growth.

In light of the evidence that pimozide and thioridazine have calmodulin antagonist activity, it is interesting that no adverse effects on the ability of the MCF-7 ER to bind [³H]estradiol or to function in the induction of PgR were observed in our studies. In calf uterus, there is evidence for calmodulin-dependent phosphorylation of the ER and regulation of estrogen receptor binding (39, 40). In MCF-7 cells, the calmodulin antagonists W-7, R24571, and trifluoperazine have been reported to inhibit the specific uptake of [³H]estradiol and decrease the number of measurable [³H]estradiol-receptor binding complexes in whole cell binding assays (41). These effects disappeared 3 h after exposure to the calmodulin antagonists. Our whole cell binding experiments showed no effect of pimozide or thioridazine on [³H]estradiol binding after 40 min, 4 h, or 5 days of drug exposure at 37°C, and they seem to rule out sustained effects of pimozide and thioridazine on the binding of estradiol to the MCF-7 ER. Moreover, our data are in agreement with Guilino *et al.* (42), who showed that trifluoperazine, W-7, and R24571

failed to compete with estradiol for MCF-7 cytosol ER binding sites.

In addition to calmodulin antagonism, pimozide and thioridazine have multiple cellular actions that may contribute to their antiproliferative effects. Pimozide and thioridazine block calcium entry through plasma membrane calcium channels in tissues such as brain, skeletal muscle, smooth muscle, and pituitary cells (43–46). Although the role of calcium in MCF-7 cell proliferation is not known, a preliminary characterization of calcium channels in MCF-7 cells has been reported (47). Pimozide has also been shown to inhibit protein kinase C activity (48). Although down-regulation of protein kinase C in MCF-7 cells by 12-*O*-tetradecanoylphorbol-13-acetate results in growth stimulation (49), not inhibition, other protein kinases may be important in MCF-7 cell growth regulation. For example, elevations in c-*myc* mRNA levels are associated with estradiol-induced growth in MCF-7 cells (50), and phosphorylation of the *myc*-encoded protein by casein kinase II has been implicated in the transduction of the mitogenic stimuli of insulin, IGF-I, and EGF to the nucleus (51–53).

Pimozide is a neuroleptic that is approved for use in the control of a neurological disorder called Tourette's disease (54). Thioridazine is a neuroleptic drug that is used to control schizophrenia and other psychoses (54). The demonstration that pimozide and thioridazine inhibit the growth of human breast cancer cell lines in tissue culture is evidence that alternative strategies for control of breast cancer proliferation *in vivo* may be found among existing drugs that have not traditionally been considered anticancer agents and provides a rational basis for the experimental use of pimozide *in vivo* to block estrogen- and polypeptide hormone-stimulated breast cancer growth. Significantly, concentrations of pimozide and thioridazine that inhibit breast cancer cell growth *in vitro* are achieved in patients receiving these drugs (43, 55) without the cytotoxic effects associated with standard chemotherapeutic regimens. Control of breast tumor growth *in vivo* by pimozide and thioridazine administration would therefore offer obvious advantages. Some adverse side effects attributable to the antidopaminergic activity of these drugs to occur *in vivo* (54). However, the incidence of Parkinson-like symptoms and tardive dyskinesia with long-term administration of thioridazine is less than observed with other phenothiazines. Elevations in serum prolactin are common in patients receiving all neuroleptic drugs. Although the data implicating prolactin in human breast tumor growth are equivocal (56, 57), prolactin receptors have been identified in 40% of human breast tumors (58). The potential for stimulation of breast tumor growth *in vivo* by pimozide and thioridazine is of concern and could affect the clinical usefulness of pimozide and thioridazine in breast cancer treatment. The concurrent use of a prolactin-lowering drug such as bromocryptine (59) in patients with prolactin receptor-positive tumors may suffice to control prolactin-stimulated tumor growth. We conclude that pimozide and thioridazine are possible candidates for further development for use in human breast cancer treatment.

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