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MECHANISMS OF GROWTH INHIBITION BY NONSTEROIDAL ANTIOESTROGENS IN HUMAN BREAST CANCER CELLS

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Summary—Treatment of MCF 7 human mammary carcinoma cells with the nonsteroidal antioestrogens, tamoxifen and clomiphene, leads to a concentration-dependent decrease in cellular proliferation rate which can be resolved into oestrogen-reversible and oestrogen-irreversible components. This became more clearly apparent when cells were treated with the 4-hydroxylated derivatives of these compounds where, because of enhanced affinty for the oestrogen receptor (ER), the dose–response curves for the two components could be separated. Thus treatment with 4-hydroxyclomiphene resulted in a distinct biphasic effect on cell growth. In the concentration range 10^{-10}–10^{-8} M, cell proliferation was inhibited in a concentration-dependent manner to a maximum of 60–70%, there was no further effect between 10^{-8} and 10^{-6} M, but at concentrations $>10^{-6}$ M there was another concentration-dependent decrease in cell growth. Studies with a series of vinyl-substituted hydroxytriphenylethylenes revealed that in the nanomolar concentration range, where the effects of the drugs could be completely negated by the simultaneous addition of oestradiol, the potency for growth inhibition was highly correlated with affinity for ER. Such data provide strong evidence that in this concentration range the growth inhibitory effects of nonsteroidal antioestrogens are mediated by the intracellular ER. In the micromolar concentration range the effects of antioestrogens are not completely reversed by oestradiol, potency is not well correlated with affinity for either ER or the antioestrogen binding site (AEBS) but the effect is cell cycle phase-specific. Furthermore, the disparity between the affinity for AEBS (0.8–3.3 nM) and the concentration of drug needed for oestrogen-irreversible growth inhibition (≥ 2.5 μM) argue against a central role for AEBS in mediating this effect.

The observation that triphenylethylene antioestrogens are calmodulin antagonists may provide some insight into potential mechanisms for this oestrogen-irreversible effect. Indeed, in identical experiments two phenothiazine calmodulin antagonists inhibited MCF 7 cell proliferation at concentrations $\geq 2.5 \times 10^{-6}$ M. Growth inhibition following administration of fluphenazine, perphenazine and triphenylethylene antioestrogens was accompanied by qualitatively similar changes in the cell cycle kinetic parameters, i.e. accumulation in G_1 phase at the expense of S phase cells. These data suggest triphenylethylene antagonism of calmodulin activated cellular processes as a potential mechanism for the oestrogen-irreversible effects of the nonsteroidal antioestrogens.

INTRODUCTION

The biology and mechanisms of action of triphenylethylene antioestrogens such as tamoxifen have been areas of intense research interest in recent years due primarily to the ability of these molecules to inhibit the growth of several hormone-dependent tumours, particularly breast cancer. In an attempt to define the molecular basis of this antitumour activity several groups have studied the effects of antioestrogens on the proliferation of human breast cancer cell lines. Lippman *et al.*[1, 2] were the first to show that tamoxifen had direct growth inhibitory effects on breast cancer cells in culture, that their effects were confined to ER-positive cells, and that the effects of tamoxifen could be negated by the simultaneous addition of oestradiol to the culture medium. It was concluded from these and other [3] data that antioestrogens exert their effects through competitive inhibition of ER-mediated events and this led to an arrest of cell cycle progression and a decreased proliferation rate, although it was apparent that cell death did occur after prolonged exposure to the drug [2, 4].

The application of analytical DNA flow cytometry to the study of antioestrogen effects on breast cancer cells has allowed more detailed analysis of antioestrogen effects on breast cancer cell proliferation kinetics. Workers in this laboratory demonstrated that tamoxifen treatment of ER-positive cells led to growth arrest which was associated with an accumulation of cells in the G_1 phase of the cell cycle [5–11], a result subsequently confirmed by others [12–15]. Furthermore, we showed that this was due to a drug-induced increase in the G_1 transit time [6] and thus the overall cell cycle time and that the effects of tamoxifen were confined to a distinct time frame in mid-G_1 phase [7]. Exposure of cells to tamoxifen outside this time frame had little or no effect on cell cycle progression [7]. Although these cell cycle changes associated with tamoxifen-induced growth inhibition were confined to ER-positive cell lines [11] it became clear from experiments where cultures were treated with both tamoxifen and oestradiol that not all the effects of tamoxifen on cell proliferation could be reversed by ostrogen, supplying strong evidence for an oestrogen-irreversible

component to the tamoxifen effect [5–11]. When further experiments were carried out with a series of different antioestrogen analogues it became apparent that while potency was generally correlated with affinity for the ER at low concentrations of the drug [8, 10, 16] this relationship did not hold in the micromolar oestrogen-irreversible concentration range [8, 16–18). In addition, treatment of MCF 7 cells with tamoxifen and ICI 145680, an analogue with identical affinity for ER but no affinity for the specific, high affinity, microsomal antioestrogen binding site (AEBS, Refs [19–21]), revealed that ICI 145680 was significantly less potent than tamoxifen over the entire concentration range studied and that the difference between the two compounds was most marked in the oestrogen-irreversible concentration range [18].

Together these data indicated the presence of at least two mechanisms of action of triphenylethylene antioestrogens on breast cancer cell proliferation, i.e. an oestrogen-reversible effect and an oestrogen-irreversible effect, and provided circumstantial evidence that in addition to effects mediated by the ER some effects of these drugs may be mediated via the AEBS. In the present paper we outline a further series of experiments aimed at developing a clearer understanding of the mechanisms involved in the control of breast cancer cell proliferation by synthetic antioestrogens *in vitro* and the relative roles of the two high affinity, intracellular binding sites for these drugs, ER and AEBS.

MATERIALS AND METHODS

Materials. Enclomiphene (*trans*-1-(*p*-β-diethylaminoethoxyphenyl)1,2 - diphenyl - 2 - chloroethylene) was obtained from Merrell National Laboratories, Cincinatti, OH, U.S.A. The 4-hydroxylated derivative of clomiphene and a series of vinyl-substituted 4-hydroxytriphenylethylene compounds were synthesised as previously described [16, 22]. Fluphenazine hydrochloride was supplied through the courtesy of Mr P. F. Levvy, E. R. Squibb & Sons Pty Ltd, Noble Park, Victoria, Australia. Other compounds were from Sigma Chemical Co., St Louis, MO, U.S.A. Stock solutions (10^{-6}–5×10^{-2} M) were prepared in ethanol, or in N,N-dimethylformamide when compounds were insoluble in ethanol, and stored at −20°C in glass vials. Procedures for the preparation of aqueous stock solutions of these compounds were as previously described [20, 21].

Trans-[N-methyl-^{3}H] tamoxifen (71–89 Ci/mmol) and [^{3}H] oestradiol-17β (90–110 Ci/mmol) were purchased from Amersham Australia, Sydney, Australia.

Estimation of affinities for ER and AEBS. Relative binding affinities (RBA) for the AEBS were determined by competitive binding assays conducted in the presence of 1 μM E_2 to eliminate interaction with ER, using MCF 7 post-mitochondrial fraction (PMF; Ref. [20]) as a source of AEBS. In outline, [^{3}H]TAM at a final concentration of 4–5 nM was incubated for 16 h at 0°C or for 1–2 h at 22°C with increasing concentrations of unlabelled ligand over the range 2.5 nM–10 μM. The incubation mixture consisted of 50 μl of 16–20 nM [^{3}H]TAM in 10 mM Tris–HCl, 25 mM KCl, 0.25 M sucrose, pH 7.4 at 22°C, buffer (TSK) containing 1 mg/ml bovine serum albumin (TSKB), 50 μl of the unlabelled competing ligand at concentrations of up to 40 μM in TSKB, and 100 μl of MCF 7 PMF in TSK. Bound and free [^{3}H]TAM were separated by charcoal–dextran adsorption. In some experiments the unlabelled competing ligands were added as ethanol stock solutions in which case instead of 50 μl of unlabelled ligand in TSKB, 40 μl of TSKB and 10 μl of ethanol stock solution were added. This concentration of ethanol had no significant effect on the determination of RBA. The use of ethanol stocks allowed high concentrations of ligands with limited solubility in aqueous solutions to be used. Stock solutions prepared in dimethylformamide could also be used in the same way. Data were plotted as per cent total or specifically bound [^{3}H]TAM vs log ligand concentration. RBA was calculated as (IC_{50} of TAM/IC_{50} of the test compound) × 100%, where IC_{50} = the concentration of ligand required to displace 50% of the specifically bound [^{3}H]TAM. The RBA of TAM for AEBS is defined as 100%.

RBA for ER, extracted from MCF 7 cell nuclei, was determined as previously described [17, 18] where the RBA for oestradiol-17β is defined as 100%.

Cell culture. Methods for culture and determination of growth inhibition and cell cycle effects were as detailed elsewhere [5–11, 17, 18]. Stock cultures of MCF 7 human mammary carcinoma cells were passaged weekly with an inoculation density of 2×10^5 cells/150 cm^2 flask into 50 ml RPMI 1640 medium supplemented with 20 mM Hepes buffer, 14 mM sodium bicarbonate, 6 mM L-glutamine, 20 μg/ml gentamicin (Essex Laboratories, Sydney, Australia), 10 μg/ml porcine insulin (CSL-Novo, Parramatta, Australia), 0.06% phenol red and 10% (v/v) fetal calf serum (FCS). All materials were from Flow Laboratories, Sydney, Australia, unless otherwise indicated.

To measure growth inhibition *in vitro*, 5×10^4 cells in exponential growth phase were plated into 25-cm^2 flasks in 5 ml of the medium described above except that the concentration of FCS was reduced to 5% (v/v). No attempt was made to remove endogenous steroids from the FCS. The same batch of FCS was used throughout this study. Twenty-four h after plating the medium was changed and the drugs added from ethanol or dimethylformamide stock solutions such that the final solvent concentration was 0.1% or 0.2% in all flasks, a concentration without effect on cell growth. After approximately

four population doublings of the control cultures (4–5 days) the cells were harvested with 0.05% trypsin, 0.02% EDTA in phosphate-buffered saline. Viable cell counts were made under phase contrast on a haemocytometer and the cells stained for analytical DNA flow cytometry with ethidium bromide–mithramycin [6]. DNA histograms were generated on an ICP22 pulse cytometer (Ortho Instruments, Westwood, MA) with excitation at 360–460 nm and fluoresence detection at greater than 550 nm. Estimates of the cell cycle kinetic parameters, i.e. the proportion of cells in the G_0/G_1, S and G_2 + M phases of the cell cycle, were calculated from the resulting DNA histograms as previously described [6].

RESULTS

Effects of antioestrogens on MCF 7 cell growth—evidence for at least two mechanisms

The effects of different concentrations of clomiphene, in the presence or absence of oestradiol, on MCF 7 cell growth are illustrated in Fig. 1. It is apparent that at doses $\leq 10^{-6}$ M the presence of oestradiol completely negates the effects of clomiphene on cell proliferation while at higher concentrations this is not the case. Such data support our earlier hypothesis of the existence of at least two mechanisms of growth inhibition by triphenylethylene antioestrogens, i.e. an oestrogen-reversible and an oestrogen-irreversible effect. This became more apparent when similar experiments were undertaken with the 4-hydroxylated derivative of this compound. 4-Hydroxyclomiphene (OH-Clom) has a 45-fold enhanced affinity for ER when compared with clomiphene (Table 1, Refs [16, 17] and as a result potency in the oestrogen-reversible concentration range is significantly enhanced resulting in a shift of the dose–response curve to the left and a distinctly biphasic response curve (Fig. 1). Thus at concentrations between 10^{-10} and 10^{-8} M, OH-Clom causes a concentration-dependent decrease in growth rate to a maximum of 60–70% growth inhibition but there was little or no further effect between 10^{-8} and 10^{-6} M. However, a further concentration-dependent decrease in cell proliferation occurred at micromolar concentrations. The effects of 10^{-10}–10^{-6} M concentrations of OH-Clom on cell growth could be completely negated by the simultaneous addition of equimolar concentrations of oestradiol to the culture medium. Effects at concentrations $\geq 2.5 \times 10^{-6}$ M were only partially reversed or unaffected by the simultaneous addition of oestradiol (data not shown). Similar data were obtained when MCF 7 cells were treated with tamoxifen and 4-hydroxytamoxifen over the same concentration range (data not shown).

Table 1. Relative binding affinities of vinyl-substituted hydroxytriphenylethylenes for ER and AEBS

Vinyl substituent	Relative binding affinity ER	AEBS
Cl	89 ± 12	119 ± 20
C_2H_5	35 ± 5	68 ± 6
Br	28 ± 4	60 ± 6
NO_2	18 ± 4	30 ± 3
CN	9 ± 3	38 ± 5
H	9 ± 1	41 ± 5

Relationship between affinity for ER and AEBS and growth-inhibitory activity

In an attempt to assess the likely role of ER and AEBS in mediating the different components of the growth-inhibitory response to antioestrogens we studied the effects of a series of vinyl-substituted hydroxytriphenylethylene compounds with varying affinities for these sites [16] on the growth of MCF 7 cells. This was a series of OH-Clom derivatives in

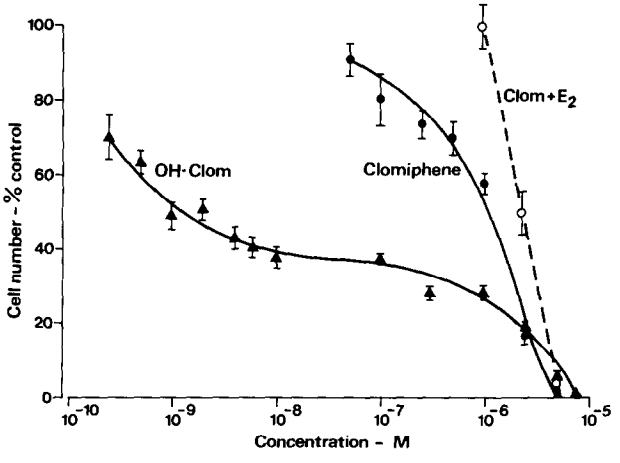

Fig. 1. Effect of 4-hydroxyclomiphene, clomiphene, and clomiphene plus oestradiol on the growth of MCF 7 breast cancer cells. After exposure of cells to varying concentrations of drug for 4 days, triplicate flasks were harvested and cell number recorded and expressed as a percentage of the cell number in untreated control flasks. The experimental design is described in detail in Materials and Methods.

Table 2. Relationship between growth inhibition and affinity for ER and AEBS

Vinyl substituent	IC_{30} (nM)	K_d—ER (nM)	IC_{90} (μM)	K_d—AEBS (nM)
Cl	0.25	0.11	4.2	0.8
C_2H_5	0.58	0.29	5.4	1.5
Br	0.85	0.36	3.6	1.7
NO_2	3.4	0.56	8.5	3.3
CN	5.4	1.11	7.1	2.6
H	20	1.11	3.8	2.4

which the vinyl Cl atom had been replaced by ethyl (Et), cyano (CN) or nitro (NO_2) groups or by a Br or H atom. The resultant compounds had a spectrum of affinities for ER and AEBS as illustrated in Table 1.

With the exception of the H-substituted compound all of these hydroxytriphenylethylenes inhibited MCF 7 cell proliferation in a distinctly biphasic fashion (Fig. 2). The relative potencies of these compounds in the nanomolar, oestrogen-reversible concentration range were in the order CL > Et > Br > NO_2 > CN > H with IC_{30} values of 0.25, 0.58, 0.85, 3.4, 5.4 and 20 nM respectively. Thirty per cent inhibition of cell growth (IC_{30}) is approximately half-maximal for the first component of the biphasic dose–response curve as illustrated in Figs 1 and 2

To investigate how this response is related to occupancy of ER, IC_{30} values were compared with apparent K_d values for ER, given that half-maximal receptor saturation occurs at a ligand concentration equal to K_d. The apparent K_d values for ER were determined from the relative binding affinities of these compounds for ER as presented in Table 1 and a K_d value of 0.1 nM previously measured at 4°C for the affinity of oestradiol for the ER of MCF 7 cells [8] and were in the order: E_2(0.10 nM) < Cl(0.11) < Et(0.29) < Br(0.36) < NO_2(0.56) < CN(1.11) = H(1.11). The relationship between K_d and IC_{30} was not linear and low affinity ligands, i.e. those with NO_2, CN and H substituents had potencies 6–18-fold lower than predicted from the corresponding K_d values for ER while the high affinity ligands (Cl, Et and Br) were only 2–2.4-fold less potent.

The order of potencies in the micromolar concentration range was significantly different from that seen at lower concentrations, supporting a different mechanism of growth inhibition (Fig. 2). In particular, the H-substituted derivative became markedly more potent at these concentrations while the NO_2 derivative was relatively less potent. IC_{90} values were in the order Br(3.6 μM) < H(3.8) < Cl(4.2) < Et(5.4) < CN(7.1) < NO_2(8.5). Potency in this range was not highly correlated with affinity for either ER or AEBS.

Changes in cell cycle kinetic parameters

Cell cycle phase distribution data were obtained in the same experiments and are summarized in Fig. 3. All compounds induced a dose-dependent decrease in the proportion of cells in S phase of the cell cycle (Fig. 3) and this was accompanied by a concomitant accumulation of cells in the G_1 phase (data not shown). As was observed with effects on cell number, dose–response curves for drug effects on S phase were biphasic with the exception of the H-substituted compound, i.e. there was a dose-dependent decrease in S phase cells between 10^{-10} and 10^{-8} M, no change between 10^{-8} and 10^{-6} M and a further concentration-dependent decrease in S phase at concentrations $>10^{-6}$ M. Drug effects at concentrations $<10^{-6}$ M were reversed by the simultaneous

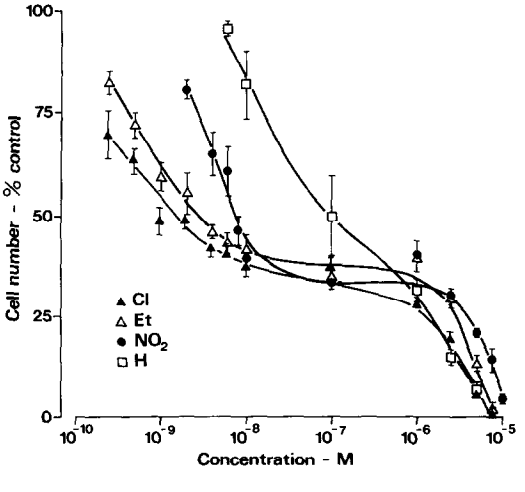

Fig. 2. Effect of vinyl-substituted hydroxytriphenylethylenes on the growth of MCF 7 breast cancer cells. The experimental design was as described in the legend to Fig. 1.

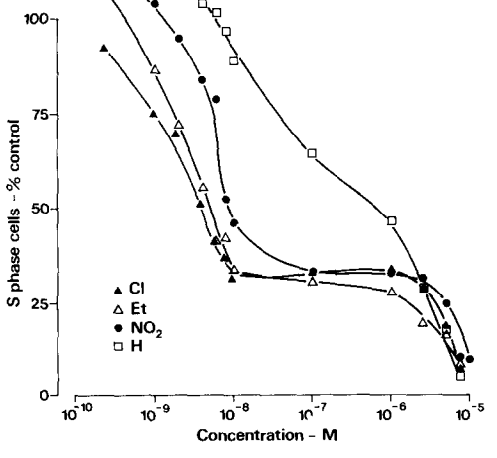

Fig. 3. Effect of vinyl-substituted hydroxytriphenylethylenes on the proportion of MCF 7 cells in the S phase of the cell cycle. Cells from the experiment described in Fig. 2 were stained for analytical DNA flow cytometry, analysed and the proportion of cells in the various phases of the cell cycle calculated as described in Materials and Methods. Data are expressed as a percentage of the S phase cells measured in untreated control cultures.

administration of estradiol while at concentrations $>10^{-6}$ M effects were incompletely reversed by estradiol (data not shown). Relative potencies in inducing decreases in the proportion of S phase cells within the concentration range 10^{-10}–10^{-8} M were in the same order as their relative potencies for growth inhibition, i.e. Cl > Et > Br > NO_2 > CN > H and were correlated with affinity for ER. It was of considerable interest that growth inhibition in the micromolar range was also accompanied by a depletion of cells in S phase and an accumulation of cells in the G_1 phase of the cell cycle. Relative potencies for depletion of the proportion of S phase cells at concentrations $>10^{-6}$ M were identical to those seen with changes in cell number, i.e. Br > H > Cl > Et > CN > NO_2, and showed no correlation with affinity for either ER or AEBS.

Effect of calmodulin antagonists on growth and cell cycle kinetic parameters

Since tamoxifen has been shown to inhibit calmodulin-dependent enzymes at micromolar concentrations [23] we investigated the effects of some calmodulin antagonists on the proliferation and cell cycle kinetics of MCF 7 cells to ascertain whether the calmodulin antagonist properties of triphenylethylene antioestrogens might explain their effects on cellular proliferation in the oestrogen-irreversible concentration range. The two compounds selected for the initial studies were the phenothiazine derivatives, fluphenazine and perphenazine because of their known affinity for AEBS [24, 25].

The phenothiazines showed potent growth inhibitory effects at concentrations $\leq 2.5 \times 10^{-6}$ M (Fig. 4) and were cytotoxic to the breast cancer cells at $1.5–2 \times 10^{-5}$ M. These effects were not reversed by the simultaneous addition of oestradiol. Although the decrease in cell proliferation induced by these compounds was accompanied by a decline in %S phase cells (Fig. 5), at any given concentration of

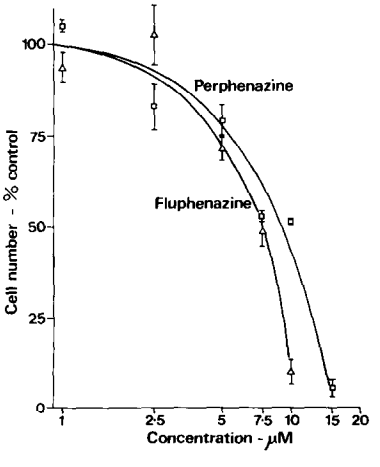

Fig. 4. Effect of two phenothiazine calmodulin antagonists on the growth of MCF 7 breast cancer cells. The experimental design was as described in the legend to Fig. 1.

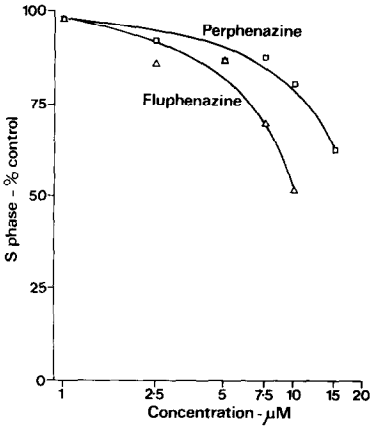

Fig. 5. Effect of two phenothiazine calmodulin antagonists on the proportion of MCF 7 breast cancer cells in S phase of the cell cycle. The experimental design is as described in Figs 1 and 3 and data are expressed as a percentage of the S phase cells measured in untreated control cultures.

these drugs the decline in cell numbers was considerably more marked than the decrease in %S phase cells. This is best exemplified at the highest concentrations presented in Figs 4 and 5 where a decrease in cell number of 90% or greater was accompanied by a decline in the %S phase cells of 40–50%. Such an effect is typical of the effects of triphenylethylene antioestrogens on MCF 7 cell proliferation when they are administered in the presence of sufficient oestradiol to eliminate their ER-mediated effects (Watts and Sutherland, unpublished observations).

DISCUSSION

In previous publications from this laboratory it has been demonstrated that nonsteroidal antioestrogens inhibit the growth of human breast cancer cells in culture by both oestrogen-reversible and oestrogen-irreversible mechanisms [5–11, 17, 18]. This has been confirmed for clomiphene in the present study. The oestrogen-irreversible effects are invariably observed at micromolar concentrations but in the case of the nonhydroxylated antioestrogens, tamoxifen and clomiphene, the oestrogen-reversible effects are also apparent in the low micromolar range precluding clear distinction of these two effects in the absence of oestradiol. The use of hydroxylated antioestrogens with their enhanced affinity for ER, resulted in markedly increased sensitivity of MCF 7 cells to the growth inhibitory effects of antioestrogens in the oestrogen-reversible concentration range and this shift in the dose–response curves allowed resolution of two distinct concentration-dependent processes (Figs 1 and 2).

Measurement of growth, in the presence and absence of oestradiol, demonstrated that the growth inhibitory effects of hydroxylated triphenylethylene antioestrogens apparent at nanomolar and low

micromolar concentrations, i.e. ≤2.5 μM, were reversed by oestrogen while those at higher concentrations were not [16, 26]. Thus these new data with hydroxylated triphenylethylene antiestrogens confirmed our earlier proposition of the existence of at least two mechanisms of growth inhibition of antioestrogens on breast cancer cells in culture; one of which is reversed by oestrogen and another which is not. The separation of the concentration dependency of these two phenomena suggests that they may be mediated by separate molecular processes.

The good correlation between affinity for ER and potency amongst the group of vinyl-substituted compounds in the nanomolar, oestrogen-reversible concentration range is in agreement with previous studies with a spectrum of different antioestrogens [8, 10, 26], and supports the view that this is an ER-mediated event.

The molecular basis of the oestrogen-irreversible mechanism has yet to be defined. We originally speculated that this mechanism may be mediated, at least in part, by the other high-affinity intracellular binding site for this group of drugs, the AEBS [17, 18]. However, the data presented here with the vinyl-substituted antioestrogens failed to show a significant correlation between relative affinity for AEBS and potency in the oestrogen-irreversible range. Furthermore, while affinities for AEBS were 1–5 nM, the concentrations of drug required for half-maximal inhibition of growth in the oestrogen-irreversible range were at least 1000-fold greater. Such a result appears incompatible with AEBS directly mediating the oestrogen-irreversible effects of antioestrogens.

In addition to binding to AEBS, the triphenylethylene antioestrogens have been shown to bind to other intracellular binding sites with affinities in the micromolar range. These include: calmodulin [23], cytochrome P-450 [27], histamine- [28], dopamine- [29] and muscarinic-receptors [30]. Since tamoxifen is a calmodulin antagonist at micromolar concentrations [23] and trifluoperazine and other calmodulin antagonists inhibit breast cancer cell growth [31] and induce G_1 arrest in CHO-K1 cells [32] it is possible that the inhibition of calmodulin-activated cellular processes by nonsteroidal antioestrogens could explain their effects on cell proliferation in the oestrogen-irreversible concentration range. This is supported by the data presented here where we were able to show that two phenothiazine calmodulin antagonists inhibit MCF 7 cell growth when administered at micromolar concentrations and this growth inhibition was accompanied by qualitatively similar changes in cell cycle kinetic parameters to those seen with similar concentrations of antioestrogens in the presence of oestradiol (Watts and Sutherland, unpublished data).

Although phenothiazines have many properties in addition to their calmodulin antagonist activity our recent observation that the more specific calmodulin antagonist, R24571, has qualitatively similar effects on MCF 7 cell proliferation to those reported here for fluphenazine and perphenazine support the view that the calmodulin antagonist properties of triphenylethylene antioestrogens may contribute to their effects in the oestrogen-irreversible concentration range. Future detailed experimentation with a broader range of calmodulin antagonists in several breast cancer cell lines will be required before this hypothesis can be adequately tested.

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