ABSTRACT

Tamoxifen and other structurally related nonsteroidal antiestrogens possess properties in addition to their estrogen antagonist activity including inhibition of both calmodulin and protein kinase C. The present studies were designed to test whether the estrogen-reversible (estrogen receptor mediated) and estrogen-irreversible effects of nonsteroidal antiestrogens on cell cycle progression in vitro were mediated at the same or different points within the cell cycle and if the estrogen-irreversible effects coincided temporally with that of a calmodulin antagonist, R24571.

Initial experiments investigated the effects of ICI 164384, a pure estrogen antagonist, on proliferation kinetics in asynchronous cultures of MCF-7 human breast cancer cells. At concentrations >1 nM ICI 164384 significantly reduced growth rate while at ≥250 nM, ICI 164384 completely arrested growth after the first 24 h of exposure. Concentrations up to 5 μM failed either to cause more profound effects on growth or induce cytotoxicity. Growth inhibition was associated with a decrease in the proportion of S phase cells and an accumulation of cells in G1 phase, and was completely reversed by the simultaneous addition of equimolar estradiol.

In order to identify the points of action within the cell cycle of ICI 164384, and the estrogen-reversible and estrogen-irreversible components of the nonsteroidal estrogen antagonist, hydroxyxilomiphene, and the calmodulin antagonist, R24571, experiments were undertaken with MCF-7 cells synchronized by mitotic selection. The mean point of action was assessed by delaying addition of the drugs for increasing time periods following mitotic selection and using DNA flow cytometry to determine the proportion of the population affected by drug administration at a specific time within G1 phase. These studies showed that sensitivity to ICI 164384 was restricted to the early part of G1 phase and that the mean time of action was 4.9 h after the beginning of G1 for this pure estrogen antagonist. The mean times of action of the estrogen-reversible (4.1 h into G1 phase) and estrogen-irreversible (4.1 h) mechanisms of action of hydroxyxilomiphene, and R24571 (4.0 h), all appeared to be within a similar time frame in early to mid G1 phase.

It is concluded that ICI 164384 inhibits breast cancer cell proliferation by inducing a transition delay in G1 phase and that the point of action of this pure estrogen antagonist in early G1 phase is indistinguishable temporally from that of nonsteroidal antiestrogens and calmodulin antagonists.

INTRODUCTION

The nonsteroidal antiestrogens, of which tamoxifen is the most studied molecule, are a group of derivatives of the synthetic estrogen, triphenylethylene. These molecules display a diversity of biological properties and behave as estrogen agonists, estrogen antagonists, or partial agonists/partial antagonists depending upon the species, tissue or response parameter under study. In a number of hormone-responsive tumors, particularly breast carcinoma, nonsteroidal antiestrogens possess potent antitumor activity. Because of this property and the low incidence of side-effects when administered to patients, one of these agents, tamoxifen, has become the treatment of choice for hormone-responsive human breast cancer (1–5).

The molecular basis of the antitumor activity of tamoxifen has not been fully elucidated but has been the subject of extensive study especially in human breast cancer cell lines in culture. Early studies revealed that the effects of tamoxifen on breast cancer cell proliferation were confined to ER-positive cells and that these effects could be reversed by the simultaneous or subsequent addition of estradiol to the culture medium (6–8). These observations led to the general belief that tamoxifen acted simply as an estrogen antagonist with competitive inhibition at the level of the ER. More extensive studies in this laboratory revealed that tamoxifen and its metabolites had both estrogen-reversible and estrogen-irreversible effects on proliferation in ER-positive breast cancer cell lines. These effects were accompanied by distinctive changes in cell cycle kinetic parameters and were, in turn, different from the observed effect of high drug concentrations on inhibition of proliferation in ER-negative cell lines (9–14). Such data raised the possibility that at least in vitro, tamoxifen had antiproliferative activity additional to that attributable to its estrogen antagonist properties. Although several other nonsteroidal antiestrogens shared these properties of tamoxifen in vitro (15–18) the contribution, if any, of mechanisms other than estrogen antagonism to the antitumor effects of tamoxifen in vivo has yet to be assessed. Attempts to address this issue both in vitro and in vivo have been hampered by the lack of molecules with pure antiestrogenic activity. However, in 1987 a new series of steroidal antiestrogenic molecules was described, which, unlike the nonsteroidal counterparts, failed to demonstrate estrogen agonist activity in a wide variety of experimental systems (19–21). These unique compounds have provided the tools necessary to distinguish the antiproliferative properties of tamoxifen that are due to estrogen antagonism from those that are independent of its estrogen antagonism. Some in vitro experiments aimed at distinguishing these effects are reported here.

Tamoxifen is now known to bind with relatively high affinity to a number of intracellular proteins in addition to the ER. These include: a specific high affinity antiestrogen binding site (22–24), calmodulin (25), protein kinase C (26), cytochrome P450 (27), and muscarinic- (28), dopamine- (29), and histamine-receptors (30). Of these potential mediators of tamoxifen action, independent of the ER, calmodulin antagonism was chosen for investigation in this study. Calmodulin antagonists have been shown to inhibit proliferation of a variety of cell types (31–34) including breast cancer cells (35, 36). The growth arrest has been attributed to sites of action in early G1 (33, 37) and blockade near the G1/S boundary (32, 37). Furthermore, preceeding calmodulin antagonists with tamoxifen in vitro and in vivo have been hampered by the lack of molecules with pure antiestrogenic activity. However, in 1987 a new series of steroidal antiestrogenic molecules was described, which, unlike the nonsteroidal counterparts, failed to demonstrate estrogen agonist activity in a wide variety of experimental systems (19–21). These unique compounds have provided the tools necessary to distinguish the antiproliferative properties of tamoxifen that are due to estrogen antagonism from those that are independent of its estrogen antagonism. Some in vitro experiments aimed at distinguishing these effects are reported here.
liminary experiments from this laboratory\(^1\) showed that the inhibition of breast cancer cell proliferation \textit{in vitro} by two phenothiazine calmodulin antagonists was accompanied by changes in cell cycle kinetic parameters qualitatively similar to those seen with nonsteroidal antiestrogens, \textit{i.e.}, accumulation of \(G_1\) phase cells at the expense of \(S\) phase cells. These data, taken together with the correlation between calmodulin antagonism and estrogen-irreversible growth inhibitory potency in a series of agents including both triphenylethylene antiestrogens and calmodulin antagonists demonstrated using MCF-7 cells \(^{36}\), provide support for the hypothesis that some of the cell cycle changes induced by antiestrogens may be attributable to calmodulin antagonism.

Previously published experiments aimed at identifying the point of action of antiestrogens within the cell cycle suffer from a number of limitations. Studies with synchronous populations of MCF-7 cells showed that tamoxifen inhibits cell cycle progression within a distinct time-frame in early to mid \(G_1\) phase \(^{11}\). However, because these experiments were conducted with micromolar concentrations of tamoxifen it is unclear whether the effects observed at the highest concentrations were due entirely to the estrogen antagonist properties of tamoxifen. The only other study \(^{39}\) employed the semiquantitative technique of chromosome condensation to document an effect in early \(G_1\) phase. The availability of ICI 164384 has allowed precise identification of effects on MCF-7 breast cancer cell cycle progression due to estrogen antagonism and facilitated a comparison with the estrogen-irreversible effects of nonsteroidal antiestrogens and those of calmodulin antagonism.

MATERIALS AND METHODS

Reagents. Insulin was purchased from CSL-Novo, Parramatta, Australia; other tissue culture materials were obtained from Flow Laboratories, Sydney, Australia. ICI 164384 (19–21) was from ICI Pharmaceuticals Division, Macclesfield, UK. Hydroxyclomiphene was synthesised as previously described \(^{40}\) and donated by Dr. P. Rueinitz, College of Pharmacy, University of Georgia, Athens, GA. Other reagents with the exception of mithramycin (Pfizer, Sydney, Australia) were purchased from the Sigma Chemical Co. (St. Louis, MO).

Tissue Culture. Stock cultures of MCF-7 cells, originally obtained from Dr. C. McGrath, Meyer L. Prentis Cancer Center, Detroit, MI, in their 299th passage, were maintained as previously described \(^{10}\) in RPMI 1640 medium containing 0.06% phenol red \[a known estrogenic compound with growth stimulatory activity for these cells \(41)\] and supplemented with 10% fetal calf serum, 6 mm l-glutamine and 10 µg/ml porcine insulin. Under these conditions MCF-7 cells grow maximally and are ideally suited for studies on growth inhibitors.

Growth inhibition of asynchronous cultures was measured by plating 5 x 10⁴ cells in exponential growth phase into T-25 tissue culture flasks in 5 ml of the same medium used for stock cultures but with the concentration of fetal calf serum reduced to 5%. The following day, when the cell number had approximately doubled, either ICI 164384, E₂ or both were added as described below. After approximately four population doublings of the control cultures, \textit{i.e.}, 5–6 days, or more frequently, triplicate flasks were harvested with 0.05% trypsin-0.02% EDTA in Ca²⁺-Mg²⁺-free phosphate buffered saline (1.5 mm K₂HPO₄·8.1 mm Na₂HPO₄·2.7 mm KCl·140 mm NaCl). Viable cells were counted using a hemocytometer under phase-contrast microscopy. The cells from each treatment group were then pooled and stained for later DNA analysis.

Synchronization by mitotic selection was achieved as previously described \(^{11}\). Mitotic cells were harvested at 4-h intervals from Day 2 or 3 cultures and plated into T-25 flasks in 5 ml fresh warm medium made as above but with 5% fetal calf serum. Each harvest, from 16 T-
progression is not complete (16, 17). We hypothesized that if the exposure of synchronized cells to a compound which exerts its effects at a specific point in the cell cycle kinetic parameters were apparent after 24 h, maximal by 3 days (at which time decreases in %S phase of up to 70-80% were observed), and maintained over a further 3-day exposure. Concentrations of 10 or 100 nM ICI 164384 administered in the simultaneous presence of equimolar E2 were ineffective in reducing either the growth rate or percentage of cells in S phase (data not shown).

Hydroxyclomiphene has qualitatively similar effects on cell growth and phase distribution when tested under the same experimental conditions (Fig. 1, inset). The ability of estrogen to reverse the growth inhibition differs in the two portions of the biphasic dose-response curve: at concentrations <1 μM hydroxyclomiphene effects are wholly reversed by the simultaneous addition of E2, while at higher concentrations they are incompletely reversed (17). Thus at a concentration of 7.5 μM, used in the experiments with synchronized cells, hydroxyclomiphene would exert both the E2-reversible and the E2-irreversible components of growth inhibition of nonsteroidal antiestrogens.

Determination of Times of Action within the Cell Cycle. We next undertook experiments designed to identify more precisely the point of action within G1 phase of ICI 164384, and of hydroxyclomiphene, and the calmodulin antagonist R24371. The experimental design illustrated in Fig. 2 was based on the following reasoning: if the exposure of synchronized cells to a compound which exerts its effects at a specific point in G1 is delayed until the cells have completed part of G1, a decreasing proportion of the population will be susceptible to its action if the delay is sufficient to allow some cells to progress past the last sensitive point. Those cells which, by virtue of their position later in G1, are no longer sensitive, will continue into S phase. If the population is then harvested at some later time which allows distinction to be made between those cells which have progressed into S phase and those retained in G1, it is possible to determine the relative proportion of sensitive cells at various times within G1. Half maximal inhibition would be expected to occur when the mean age of the cells at the time drug treatment began was equal to the time of action, i.e., when half the population was before, and half the population after, the sensitive time.

Control cultures harvested 1-24 h after mitotic selection demonstrated a high degree of synchrony, in that the G1 fraction was initially 80-90% and after 6-8 h fell to a minimum of 25-30% (data not shown). The mean time to exit G1 for control cells set up in parallel with the treated cultures was 8.7 ± 0.3 h (mean ± SE, N = 10). On the basis of these experiments 16 h after mitotic selection was chosen as an appropriate time to determine the number of cells retained in G1 following drug treatment, since at that time cells initiating DNA synthesis had progressed into late S phase (enabling good discrimination between sensitive and insensitive cells) but had not completed mitosis (Fig. 3 and ref. 11). DNA histograms of cells exposed to 7.5 μM hydroxyclomiphene following delays of up to 6 h after, and harvested 16 h after, mitotic selection are shown in Fig. 3. Maximal inhibition of progression into S phase occurred when drug treatment began less than 2 h after the beginning of G1. When treatment was commenced thereafter, decreasing numbers of cells were affected until after a 6 h delay very few cells were retained in G1. At this concentration, hydroxyclomiphene caused a slight delay in transit through S phase, in addition to the primary effect of arrest in G1, as evidenced by the lower DNA content of the cohort of cells in S phase compared with control histograms (Fig. 3).

The reduction in sensitivity to hydroxyclomiphene apparent with delayed commencement of drug treatment within G1 oc-
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Fig. 2. DNA histograms of cells treated with 7.5 μM hydroxyclomiphene. The experimental design was as described in Fig. 1. Each histogram contains data from 16,000 to 19,000 cells and has a coefficient of variation between 3.1 and 3.6%.

ocurred as the duration of exposure to the drug was reduced from 16 to 10 h. However it is not simply due to decreased total exposure time. Fig. 4 presents the results of experiments in which 7.5 μM hydroxyclomiphene was administered as a 3-h pulse beginning 0, 3, or 6 h after mitotic selection. Again the sensitivity of the cells was reduced as they moved through G1, with the pulse commencing 6 h after selection having a significantly smaller effect (P < 0.025) than that commencing at 0 h. This indicates that cells in late G1 are indeed relatively insensitive to 7.5 μM hydroxyclomiphene.

We then sought to determine the precise time of action within G1 by making observations 1 h apart, up to a maximum of 8 h after mitotic selection. The number of cells blocked in G1 was obtained by subtraction of the %G1 of parallel control samples from the %G1 of the drug-treated sample. Fig. 5 shows the reduction in numbers of such cells with increasing delay of administration of hydroxyclomiphene in the presence or absence of 1 μM E2. Under both sets of experimental conditions half-maximal retention of cells in G1 occurred at 4.1 h, establishing the temporal identity of the E2-reversible and E2-irreversible actions of this drug.

Similar experiments were performed using 100 nM ICI 164384. In the presence of equimolar E2, ICI 164384, failed to increase the %G1 compared to untreated control cells (Fig. 6).

Although the effect is more modest than that achieved with 7.5 μM hydroxyclomiphene it can be concluded firstly that the maximum effect is seen only for cells early in G1 while late in G1 most cells are insensitive to ICI 164384, and secondly that under these experimental conditions the growth arrest induced by this compound is abrogated by the simultaneous addition of
equimolar E₂. The half-maximal inhibition of initiation of DNA synthesis occurred 4.9 h after the beginning of G₁.

To test the hypothesis that the calmodulin antagonist activity of tamoxifen provides a potential mechanism for mediation of the E₂-irreversible and therefore putatively non-ER-mediated actions of nonsteroidal, triarylethylene antiestrogens, the time of action of the potent calmodulin antagonist, R24571, was determined using the same protocol. A concentration of 2.5 μM was chosen on the basis of previous experiments using asynchronous cultures, which showed a degree of growth inhibition similar to that of 7.5 μM hydroxylomiphene, and concomitant increases in %G₁ phase cells. The results are presented in Fig. 7 where again sensitivity to the agent declined with transit through G₁, and the half maximal effect occurred at 4.0 h. DNA histograms of R24571-treated cells showed that this concentration of R24571 did not produce evidence of a second block in early S phase (data not shown), as has been observed with some calmodulin antagonists in other cell types (37).

**DISCUSSION**

There is accumulating evidence to suggest that nonsteroidal antiestrogens have both estrogen-reversible and estrogen-irreversible, but cell cycle specific, components to their action as growth inhibitory agents in vitro (9–18, 36, 38). Detailed cell kinetic studies employing synchronized cells have been restricted to concentrations of tamoxifen in the micromolar range (11). The possibility that at these high concentrations the predominant effect is not mediated through the ER prompted an examination of the kinetics of cells exposed to a pure estrogen antagonist, specifically to determine its point of action within the cell cycle and to establish the relationship between this and the observed effects of nonsteroidal estrogen antagonists.

The present study confirms earlier reports (19, 20) that the pure estrogen antagonist ICI 164384 inhibits the proliferation of human breast cancer cells in culture. The observed pattern of cell cycle kinetic effects is similar to that seen with the nonsteroidal antiestrogens and their hydroxylated derivatives (compare refs. 12, 14, 16, 17, 39, 44, 45) in inhibiting ER-positive breast cancer cell growth and is consistent with a site of action early in G₁. If this is the major site of action and the remainder of the cell cycle is relatively unaffected, the majority of the cell population will be capable of progression through the remainder of G₁, S, G₂, and mitosis and back into G₁ phase in the first cell cycle of exposure. This would account for the initial increase in cell number at control rates during the first 24 h of drug exposure as seen in Fig. 1. However, few of the daughter cells will commence DNA synthesis because of their inability to overcome the block in early G₁, resulting in a sharp drop in %S phase and in population doubling time. At concentrations which are cytostatic but not cytotoxic this mechanism is entirely estrogen-reversible and therefore presumably mediated solely through the ER. Although the cell cycle correlates of the growth arrest, i.e., increase in the number of cells in G₁ phase and decrease in %S phase, are qualitatively similar to those observed using other, nonsteroidal, estrogen antagonists, and ICI 164384 is growth inhibitory over a concentration range (1–100 nM) similar to the estrogen-reversible actions of vinyl substituted hydroxytriphenylethlenes, e.g., hydroxylomiphene (16, 17), these effects are quantitatively greater for ICI 164384. This is illustrated by the observation that in the presence of 100 nM ICI 164384 the cell number is reduced by 88% compared to control, after four doublings of the untreated control population, whereas nonsteroidal antiestrogens with high affinity for the ER such as hydroxylomiphene have a maximum entirely E₂-reversible growth inhibition of 60–70% (see inset to Fig. 1). It is possible that the estrogen agonist activity of nonsteroidal antiestrogens demonstrated both in vivo (1–5), and in vitro in estrogen-free culture conditions (46, 47),

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Fig. 6. Effect of the time of exposure to ICI 164384 on the proportion of synchronized MCF-7 cells remaining in G₁ phase. Cells were exposed to 100 nm ICI 164384 in the presence (C) or absence (8) of equimolar E₂, commencing at the indicated time after mitotic selection, according to the protocol illustrated in Fig. 2. Points, mean of data from two or three separate experiments. The %G₁ of parallel control samples has been subtracted; error bars, a combination of the errors in the estimation of individual data points as described in “Materials and Methods.” The line of best fit was calculated by linear regression analysis of data between 1 and 6 h inclusive; r² = 1.00.

Fig. 7. Effect of the time of exposure to the calmodulin antagonist R24571 on the proportion of synchronized MCF-7 cells remaining in G₁ phase. R24571 was administered at a concentration of 2.5 μM as described in Fig. 2. Points, mean of two to three experiments in which the %G₁ of parallel control samples has been subtracted from each data point; bars, errors calculated as described in “Materials and Methods.” Data from 1 to 6 h have been used to calculate the line of best fit (r² = 0.98).
The temporal coincidence of anticalmodulin- and antiestrogen sensitivity and calmodulin antagonist sensitivity demonstrates a significant inverse relationship between intracellular calmodulin levels and the length of G₁ phase in transplanted tumors. This hypothesis is supported by the authors (50, 51), who noted that the blockade in G₁ observed during the first cell cycle of drug exposure of synchronous cells effectively reduced the growth fraction of the treated population and thus increased the doubling time substantially. The effect was insufficient to account for the profound growth inhibition observed with long-term treatment of asynchronous cultures. The cumulative effects of tamoxifen, which are manifested in asynchronous cultures as a progressive increase in %G₁ cells and decrease in %S phase over several days' treatment, do not provide evidence for additional mechanisms of action becoming apparent with extended exposure (9, 10, 39, 44). Rather, they suggest that the same mechanism results in additional cells leaving the cycle at each round of replication and it is likely that this is also the case for the agents examined here.

Recent data showing a significant positive correlation between the degree of calmodulin antagonism and E₂-irreversible growth inhibitory potency in MCF-7 cells (36) argue for inhibition of calmodulin-dependent processes as a likely mechanism for the E₂-irreversible growth inhibition apparent at micromolar concentrations of antiestrogens. This hypothesis is supported by the temporal coincidence of estrogen-reversible antiestrogen sensitivity and calmodulin antagonist sensitivity demonstrated in the present study, which additionally confirms the early G₁ site of action for calmodulin antagonism found using the anticalmodulin drugs W13 (37) and trifluoperazine (33). The temporal coincidence of anticalmodulin- and antiestrogen-sensitive steps has important implications for cell cycle control in hormone-sensitive cells, since it implies that there may be a process crucial to cell cycle progression common to both types of agents. Further evidence for a common mechanism is provided by the ability of EGF to reverse, at least partially, the actions of antiestrogens (48) and some calmodulin antagonists (33).

The recent impressive evidence from gene transfer experiments showing a significant inverse relationship between intracellular calmodulin levels and the length of G₁ phase in transfected cells argues strongly for a fundamental regulatory role for calmodulin in cell cycle progression (49). In view of these observations it would be interesting to know what effects, if any, estrogens and antiestrogens have on calmodulin gene expression in human breast cancer cells, since effects at this level could explain the apparent convergence of ER-mediated and calmodulin pathways. A more direct effect of calmodulin on estrogen action has been suggested by data implicating calmodulin-dependent tyrosine phosphorylation of the ER in activation of this molecule (50). In the most recent studies from this group estrogen has been shown to activate, and antiestrogens inhibit, the kinase activity and in turn conversion of ER

from a nonbinding to a hormone-binding state (51). If, as suggested by the authors (50, 51), this process is critical to estrogen action the parallels between inhibition of receptor phosphorylation by calmodulin antagonists and antiestrogens are obvious and would provide a potential explanation for the coincidence of the effects of these agents on breast cancer cell cycle progression reported here.

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Points of Action of Estrogen Antagonists and a Calmodulin Antagonist within the MCF-7 Human Breast Cancer Cell Cycle

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