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 ≥50 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, hydroxyxyclophene, 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 drug 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 hydroxyclophene, 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 include compounds that 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 P-450 (27), and muscarinic- (28), dopamine- (29), and histamine-receptors (30). Of these potential mediators of tamoxifen action 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.

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liminary experiments from this laboratory\(^5\) (38) showed that the inhibition of breast cancer cell proliferation in vitro by two phenothiazine calmodulin antagonists was accompanied by changes in cell cycle kinetic parameters qualitatively similar to those seen with nonsteroidal antiestrogens, 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 triphenylethylenic 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. Hydroxychloromiphene was synthesized 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 \(\mu\)g/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 \(\times\) 10^4 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, E\(_2\) or both were added as described below. After approximately four population doublings of the control cultures, i.e., 5–6 days, or more frequently, triplicate flasks were harvested with 0.05% trypsin-0.02% EDTA in Ca\(^{++}\)-Mg\(^{++}\)-free phosphate buffered saline (1.5 mm KH\(_2\)PO\(_4\), 8.1 mm Na\(_2\)HPO\(_4\), 2.7 mm KCl, 140 mm NaCl). Viable cells were counted using a hemacytometer 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-150 flasks set up at 1 \(\times\) 10^6 cells/flask, yielded sufficient cells to set up four to five T-25 flasks (i.e., 1–2 \(\times\) 10^6 cells/T-150) and took approximately 30 min. The time at which the flasks were placed into the incubator at 37°C following mitotic selection was designated 0 h and taken to be the commencement of G\(_1\) since DNA analysis demonstrated that almost all of the selected population had completed mitosis and entered G\(_1\) at that time (11).

**Drug Treatment.** ICI 164384, hydroxychloromiphene, R24571, and E\(_2\) were stored at \(-20°C\) as 1,000-fold concentrated stock solutions dissolved in analytical reagent grade ethanol or N,N-dimethylformamide and added directly to the culture medium so that the final vehicle concentration was no greater than 0.2%. Control flasks received vehicle alone to the same final concentration and this was shown to have no significant effect on MCF-7 proliferation when compared with control cultures without vehicle.

In general synchronized cells were exposed to each compound for a period commencing at the indicated time after mitotic selection and continuing until harvest for DNA analysis at 16 h. In some experiments, which involved treatment with 7.5 \(\mu\)m hydroxychloromiphene in 3-h pulses, the culture medium was removed at the end of the exposure time and the monolayer washed gently with two aliquots of 2 ml warm fresh medium before refeeding with 5 ml warm fresh culture medium. The cells were then returned to the incubator at 37°C and harvested 16 h after mitotic selection.

**DNA Analysis.** Harvested cells were collected by centrifugation (350 \(\times\) g \times 5 min), drained, and then stained by resuspension at a concentration of no more than 2 \(\times\) 10^7 cells/ml in culture medium to which ethidium bromide (40 \(\mu\)g/ml), mithramycin (12.5 \(\mu\)g/ml), Triton X-100 (0.2% v/v), and MgCl\(_2\) (7.5 mM) had been added (42). The samples were stored at 4°C for later flow cytometric DNA analysis, which was performed using a FACStar instrument and accompanying DNA analysis software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The DNA stained cells were illuminated with laser light at 457 nm and the resulting emission from the DNA fluorochromes measured at 585 nm (585/42 bandpass filter). The cell cycle phase fractions were estimated using a model in which a second-order polynomial is fitted to S phase (SPFIT), or for histograms of synchronized populations, using an algorithm which iteratively fits the S phase region of the DNA histogram with the sum of a number of broadened rectangles (SOBIR (43)); in the second model in addition to the phase fractions the standard deviation of the estimation of each phase is computed and this was used to indicate the error in the phase fractions.

**Data Analysis.** Where data points were obtained by subtraction of the %G\(_1\) of a control histogram from that of a treated sample, the error in the difference was calculated as the square root of the sum of the standard deviation of the control %G\(_1\) estimate, squared, and the standard deviation of the treated %G\(_1\) estimate, squared. Where the data from several experiments are pooled, the error displayed is the square root of the sum of the standard error of the mean of the pooled data, squared, and the mean errors in estimation of each individual data point, squared. The time of action of a particular agent within the cell cycle was calculated as the time corresponding to inhibition 50% of that observed when drug treatment began 1 h after mitotic selection, using the equation of a line of best fit determined by linear regression.

**RESULTS**

Growth Inhibition of Asynchronous Cells with ICI 164384 or Hydroxychloromiphene. In order to investigate the effects of the pure estrogen antagonist, ICI 164384, on MCF-7 cell proliferation kinetics exponentially growing cultures were treated with different concentrations of the drug for up to 6 days. At concentrations between 1 and 50 nm, ICI 164384 inhibited the proliferation of MCF-7 cells in a dose-dependent fashion after an approximate doubling of cell numbers during the first 24 h (Fig. 1). Concentrations up to 5 \(\mu\)M were no more effective than 50 nm, which was cytostatic after the first 24 h of exposure. There was evidence of cytotoxicity only at concentrations >5 \(\mu\)M, where cell numbers were below the drugging density. The
reduction in growth rate was preceded by a marked reduction in the proportion of cells in S and G2 + M phases and concomitant increase in the G1 fraction (data not shown). These changes in 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 reversible 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 R24571. 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-
CELL CYCLE EFFECTS OF ESTROGEN ANTAGONISTS

Fig. 4. Effect of pulsed exposure of synchronized cells to 7.5 μM hydroxyclomiphene. Treatment of mitotically selected cells began at 0, 3, or 6 h. After 3 h, exposure to the drug was terminated by removing the culture medium and washing the monolayer. The cells were refed with fresh warm medium and incubation at 37°C continued until harvest at 16 h. The %G1 of parallel control samples has been subtracted from each data point. Bars, SD in estimation of the %G1 for both treated and control histograms combined as described in “Materials and Methods.”

Fig. 5. Effect of the time of exposure to hydroxyclomiphene on the proportion of synchronized MCF-7 cells remaining in G1 phase. The experimental design is as described in Fig. 2, using 7.5 μM hydroxyclomiphene alone (○) or in the presence of 1 μM E2 (□). Points, mean of two to four individual experiments in which the %G1 of parallel control samples has been subtracted from each data point; bars, combination of errors in data points and the SE of their mean, calculated as described in “Materials and Methods.” Regression lines are calculated using data from 1 to 6 h inclusive; r² = 0.99 for both hydroxyclomiphene alone and hydroxyclomiphene plus E2.

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

occurred 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).
was chosen on the basis of previous experiments using 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 hydroxoclophene, 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 G₀ 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., hydroxyclophene (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 hydroxyclophene 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),
is also operating under the experimental conditions described here and reduces the apparent efficacy of the growth inhibitory action.

The experiments designed to identify the point of action of antiestrogens within the cell cycle show that the last point at which IC1 164384 is effective is located 4–5 h after the beginning of G1, around 4 h before initiation of DNA synthesis. This antiestrogen-sensitive step is coincident with both the E2-reversible and E2-irreversible actions of hydroxytremine. Previously published data had placed tamoxifen sensitivity within a 2 h window in early-to-mid G1 (11), and indeed data obtained using 7.5 μM tamoxifen and an identical experimental design to that detailed here6 are in close agreement with the timing observed in the present experiments.

Although 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 with 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-irreversible 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 effects of tamoxifen and antiestrogens on hormone-responsive human breast cancer in long term tissue culture. Cancer Res., 36: 4595–4601, 1976.


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CELL CYCLE EFFECTS OF ESTROGEN ANTAGONISTS


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

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