Effects of Biologically Active Metabolites of Tamoxifen on the Proliferation Kinetics of MCF-7 Human Breast Cancer Cells

in Vitro

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INTRODUCTION

Tamoxifen, a nonsteroidal antiestrogenic compound, has been widely used in the treatment of human breast cancer on account of its efficacy and relatively low toxicity (22). Although this agent has been in clinical use for over a dozen, the mechanism of its action remains incompletely understood (29). These data support the concept that estrogens and antiestrogens compete for a common event which regulates the rate of cell proliferation probably by controlling the proportion of cells entering S phase. Although it appears likely that ER is intimately involved in this regulatory process, 17β-estradiol-reversible mechanisms are also involved in antiestrogen action in vitro.

MATERIALS AND METHODS

Cells. MCF-7 cells (26) in their 299th passage were supplied by Dr. Charles M. McGrath, Meyer L. Prentis Cancer Center, Detroit, Mich., and maintained as described previously (28). Stock cells were passaged weekly with an inoculation density of 3 x 10⁶ cells/150-sq cm flask into 50 ml Roswell Park Memorial Institute Medium 1640 supplemented with 20 mw 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 14 mM sodium bicarbonate, 5 mw L-glutamine, gentamicin (20 µg/ml), insulin (10 µg/ml), and 10% fetal calf serum.

Drug Treatment. Tamoxifen [trans-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene], 4-demethyltamoxifen [trans-1-(4-β-meth-ylaminoethoxyphenyl)-1,2-diphenylbut-1-ene], and 4-hydroxytamoxifen [trans-1-(4-β-dimethylaminooetoxyphenyl)-1-(4-hydroxyphenyl)-2-phenylbut-1-ene] were supplied by I. C. I., Ltd., Pharmaceuticals Division, Macclesfield, Cheshire, United Kingdom. Stock solutions (10 mw) were prepared in analytical grade ethanol and stored at -20°. These drugs were added to culture medium as described previously (29).

17β-Estradiol was acquired and handled as described previously (28).

Growth Curves. Exponentially growing cells (5 x 10⁶) from Day 5 stock cultures (28) were inoculated into 25-sq cm flasks (Coming, N. Y.) in 5 ml medium containing 5% fetal calf serum. When cell numbers.

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ABSTRACT

The effects of two major metabolites of tamoxifen, N-demethyltamoxifen (DMT) and 4-hydroxytamoxifen (4OHT), on MCF-7 cell proliferation and cell cycle kinetic parameters were compared with those of the parent compound. All three compounds produced dose-dependent decreases in the rate of cell proliferation which were accompanied by decreases in the percentage of S- and G2-M-phase cells. 4OHT was 100- to 167-fold more potent than both tamoxifen and DMT in producing these effects, and this was correlated with their relative binding affinities (RBAs) for the cytoplasmic estrogen receptor (ER) (17β-estradiol = 100, 4OHT = 41, tamoxifen = DMT = 2). At doses ≤2.5 µM, these effects were completely reversed by 17β-estradiol, but the required 17β-estradiol:antiestrogen concentration ratios differed, i.e., 1:10 to 1:1 for 4OHT compared with 1:1000 to 1:100 for tamoxifen and DMT. Although the concentrations of 17β-estradiol required for reversal were related to affinity of the metabolite for ER, they were 5- to 20-fold lower than predicted from the measured RBAs. When the rate of cell proliferation was measured over a range of concentrations of antiestrogen, in the presence or absence of 17β-estradiol, it was highly correlated (r² = 0.96) with the percentage of S-phase cells. In addition to these 17β-estradiol-reversible events, all three compounds caused 17β-estradiol:inhibitory cytoxicity at higher concentrations (≥7.5 µM DMT and 4OHT, 10 µM tamoxifen). The order of potency of producing this effect was DMT > 4OHT > tamoxifen, which correlated with neither the RBAs for the ER nor the RBAs for the high-affinity microsomal antiestrogen binding site.

These data support the concept that estrogens and antiestrogens compete for a common event which regulates the rate of cell proliferation probably by controlling the proportion of cells entering S phase. Although it appears likely that ER is intimately involved in this regulatory process, 17β-estradiol-reversible mechanisms are also involved in antiestrogen action in vitro.

tumor tissues of patients treated with tamoxifen is DMT, with 4OHT being present at much lower concentrations (1, 7, 13, 25). Prompted no doubt in part by the early misidentification of the major metabolite as 4OHT, there has been considerable interest in the antiestrogenic and antitumor activity of 4OHT (2, 3, 6, 15, 16, 33). In contrast, there have been very few studies on the biological activity of DMT (6, 33).

In the present study, we have attempted to answer the following questions. Do the metabolites DMT and 4OHT differ from tamoxifen in their actions on the proliferation and cell cycle kinetics of human breast cancer cells? What are the relative potencies of these 3 compounds in their actions on such cells in vitro? What therefore is the likely contribution of each to the antitumor activity observed in vivo? Can the relative potencies of these compounds in producing these effects be related to their RBAs for the ER or the more recently described high-affinity antiestrogen binding site (23, 30)?

It has previously been shown in this laboratory that tamoxifen has growth-inhibitory effects on the MCF-7 human breast cancer cell line and that not all of these effects are reversed by simultaneous treatment with 17β-estradiol (27, 28). These inhibitory effects were shown to be accompanied by accumulation of cells in the G1-G0 phase of the cell cycle (27, 28), due mainly to an increase in the proportion of "slowly cycling" cells (28, 32). The MCF-7 cell line forms a particularly suitable experimental model to compare the effects of tamoxifen and its metabolites since it has previously been shown that metabolism of tamoxifen is negligible in these cells in vitro (3, 14).
Chart 1. Effect of tamoxifen (A), DMT (B), and 4OHT (C) on the growth of MCF-7 cells. After 30 hr of exponential growth, cells were treated with tamoxifen, DMT, or 4OHT at concentrations of 0 (.), 0.01 (C), 0.1 (A), 0.5 (D), 2.5 (.), 5 (.), 7.5 (.), or 10 μM (O). Treated medium was changed daily thereafter, and cells were harvested and counted at 96 and 144 hr. D, cell counts at 144 hr for tamoxifen (.), DMT (.), and 4OHT (.). Points, mean of triplicate flasks from 2 experiments; bars, S.E. (n = 6). Inset, 2 to 10 nM 4OHT compared with 1 μM tamoxifen at 120 hr; columns, mean of quadruplicate flasks; bars, S.E.

Reached approximately 1 x 10⁶/flask, the medium was replaced with fresh medium containing antiestrogen with or without 17β-estradiol. The experimental medium was changed daily thereafter. Cells were harvested with 0.05% trypsin:0.02% EDTA (Flow Laboratories, Ryde, New South Wales, Australia), and viable cells were counted under phase-contrast on a hemocytometer or in a Model ZBI Coulter Counter (Coulter Electronics, Hertfordshire, United Kingdom) after 2 and/or 4 doubling times of the control population.

Flow Cytometry. Harvested cells were stained for DNA flow cytometry as previously described (31) and analyzed on an ICP22 pulse cytometer (Ortho Instruments, Westwood, Mass.). Estimates of the proportions of cells in G0-G1, S, and G2-M phases of the cell cycle were computed from the resulting DNA histograms (21).

Isotopes. [3H]Estradiol (100 Ci/mmol) was purchased from Amersham International, Amersham, Buckinghamshire, United Kingdom, and [3H]-tamoxifen (71 Ci/mmol) from New England Nuclear, Boston, Mass. [3H]-4OHT (42 Ci/mmol), a 1:1 mixture of the cis and trans isomers, was supplied by I. C. I., Ltd., Pharmaceuticals Division, and trans-4-[3H]-hydroxytamoxifen was prepared from this material by a modification of the high-performance liquid chromatography method of Foster et al. (10) as follows. Isotope was applied to a Brownlee RP-18 reverse-phase 10-μm (22 × 0.7-cm) column and eluted in 75% methanol:25% water:0.1% diethylamine at 2.0 ml/min using a Waters Associates M45 solvent delivery system. Column eluent was monitored by absorbance at 254 nm and by radioactivity. The retention time of the cis isomer was 24% longer than the trans isomer. The radioactive peak corresponding to trans-4-hydroxytamoxifen was collected, dried under nitrogen, and redissolved in ethanol. Aqueous solutions of trans-4-[3H]hydroxytamoxifen were prepared by drying a portion of the ethanolic solution under nitrogen and redissolving in 10 mM Tris:1.5 mM EDTA buffer, pH 7.4 (Tris:EDTA buffer) containing 0.2% bovine serum albumin. These solutions were used within 24 hr of preparation.

Determination of Binding Parameters. The cells used for binding studies were obtained by plating 2 x 10⁶ logarithmically growing cells.
into 150-sq cm flasks in 50 ml medium and harvesting 6 to 7 days later when cell numbers were 3 to 4 x 10^7 cells/flask. Cells were harvested with 1 mM EDTA in Dulbecco's phosphate-buffered saline (140 mM NaCl:2.7 mM KCl:8.1 mM Na_2HPO_4:1.5 mM KH_2PO_4, pH 7.3) and washed once with phosphate-buffered saline and once with Tris:EDTA buffer, each wash being followed by centrifugation at 800 x g for 10 min. The cells were homogenized in Tris:EDTA buffer (3 x 10^7 cells/ml) with a Teflon:glass homogenizer, and a cytosol was prepared by centrifugation at 135,000 x g for 1 hr.

Endogenous estrogens were removed from the cytosol by a 30 min incubation with 0.1 volume 5% charcoal:0.5% dextran followed by a 10-min centrifugation at 1500 x g.

**RBAs.** To determine the ability of various ligands to compete for ER, a constant concentration of [³H]estradiol (0.5 nM) was incubated for 16 hr at 4° with increasing concentrations of unlabeled ligand. Data were plotted as percentage of tracer bound versus log ligand concentration, and RBAs were calculated using the method of Korenman (18).

**Apparent Affinity Constants.** Saturation analysis of binding to ER using increasing concentrations of trans-4-[³H]hydroxytamoxifen (0.05 to 4 nM), [³H]tamoxifen (0.25 to 15 nM), or [³H]estradiol (0.05 to 2 nM) was performed by incubating 100 µl cell extract with 50 µl isotope and 50 µl buffer or 50 µl unlabeled ligand (4 µM) for 16 to 20 hr at 4°. Bound and unbound ligand were separated by a 30-min incubation at 4° with 500 µl 0.5% charcoal:0.05% dextran followed by centrifugation at 1500 x g for 10 min at 4°. Data were analyzed by the method of Scatchard following correction for nonspecific binding (5).

## RESULTS

**Effects of Tamoxifen Metabolites on Cell Proliferation.** The effects of tamoxifen and the 2 metabolites, DMT and 4OHT, on the growth of MCF-7 cells in medium supplemented with 5% FCS are shown in Chart 1. Control cells grew exponentially with a doubling time of about 28 hr. Each of these 3 compounds caused a dose-dependent decrease in the rate of cellular proliferation. The lowest concentration of tamoxifen and DMT found to produce a significant decrease in cell numbers after 114 hr of treatment was 100 nM, and these 2 compounds were approximately equipotent over the concentration range 100 nM to 5 µM (Chart 1D). In contrast, 4OHT was greater than 50-fold more potent at the lower concentrations, with 10 nM 4OHT producing more inhibition than 500 nM tamoxifen or DMT (Chart 1, C and D). The potencies of all 3 compounds in inhibiting cell growth tended to converge at concentrations of 2.5 to 5 µM, and at higher concentrations cytotoxicity was observed with DMT and 4OHT (7.5 to 10 µM), both causing a rapid decline in cell numbers which was significantly greater than the cytotoxicity produced by 10 µM tamoxifen (Chart 1).

To further define the relative potencies of 4OHT and tamoxifen, 1 µM tamoxifen was compared with 4OHT over a concentration range of 2 to 10 nM (Chart 1D, inset). This dose of tamoxifen inhibited cell proliferation to the same extent as 6 to 10 nM 4OHT, i.e., 4OHT was 100- to 167-fold more potent than tamoxifen, under these experimental conditions.

**Reversibility by Estradiol.** In order to assess the ability of 17β-estradiol to reverse the inhibitory effects of tamoxifen and its 2 metabolites on MCF-7 cell proliferation, cells were treated simultaneously with 17β-estradiol and tamoxifen, DMT, or 4OHT at relative concentrations of 1:1000 to 1:1 (Chart 2).
Under these conditions, 17β-estradiol alone produced either a small but significant increase [16 ± 2% (S.E.); n = 36; p < 0.025] in cell numbers over control when administered at $10^{-10}$ to $2.5 \times 10^{-7}$ M or caused a small decrease (24 ± 6%; n; 6; p < 0.05) at a dose of $5 \times 10^{-6}$ M.

The effects of tamoxifen and DMT (0.1 to 2.5 μM) were fully reversed by 1,000-fold lower concentrations of 17β-estradiol and, indeed, for 0.1 and 0.5 μM tamoxifen and DMT, simultaneous administration of 17β-estradiol at relative concentrations of 1:100 to 1:1 produced a small but significant stimulation of cell proliferation (Chart 2, A and B) not exceeding that seen with the same concentrations of 17β-estradiol alone. Almost complete reversal of the effect of 5 μM tamoxifen was produced by 100- and 10-fold lower concentrations of 17β-estradiol (50 to 500 nM), but no reversal was seen with equimolar 17β-estradiol (Chart 2A). Inhibition produced by 5 μM DMT was incompletely reversed by 1000- to 10-fold lower concentrations of 17β-estradiol (50 to 500 nM), and the addition of equimolar 17β-estradiol caused further inhibition of cellular proliferation (Chart 2B).

In contrast, the inhibitory effect of 0.1 and 0.5 μM 4OHT was fully reversed only by equimolar or 10-fold lower concentrations of 17β-estradiol (Chart 2C). Complete reversal of the growth-inhibitory effect of 2.5 μM 4OHT was not apparent at any concentration of 17β-estradiol tested; however, 0.25 μM 17β-estradiol resulted in partial reversal to approximately 70% of control cell numbers. In the presence of 5 μM 4OHT, equimolar or 10-fold lower concentrations of 17β-estradiol caused inhibition additional to that produced by 5 μM 4OHT alone (Chart 2C).

This latter effect was seen at all concentrations of antiestrogen which induced a decline in cell numbers, i.e., no reversal was apparent at any 17β-estradiol concentration tested, and at some concentrations 17β-estradiol augmented this cytotoxic effect of antiestrogens (data not shown).

Cell Cycle Parameters. Flow cytometric analysis of drug-treated cells demonstrated that all 3 compounds caused a dose-dependent reduction in the proportion of cells in S phase and, to a lesser extent, in G2-M phase of the cell cycle, with a corresponding increase in the proportion of cells in G0-G1 phases (Chart 3). Over the concentration range shown (0.01 to 5 μM), 4OHT was at least 50-fold more potent than both tamoxifen and DMT in causing these changes in cell cycle parameters, but tamoxifen and DMT were approximately equipotent.

In order to assess the estrogen reversibility of these effects, cells were treated simultaneously with 17β-estradiol and antiestrogen at a relative concentration ratio of 1:10, and cell cycle kinetic parameters were calculated after approximately 4 doublings of the control population. Under these conditions, 17β-estradiol alone ($10^{-9}$ to $5 \times 10^{-7}$ M) caused a small but significant rise in the proportion of cells in S phase (5 ± 1%; n = 45; p < 0.01). Complete reversal by 17β-estradiol of the drug induced effects on percentage of S-phase cells was seen with concentrations up to 2.5 μM tamoxifen, 0.5 μM DMT, and 0.5 μM 4OHT, with small increases over control being seen for tamoxifen (0.01 to 2.5 μM) plus 17β-estradiol and for DMT (0.01 and 0.1 μM) with 17β-estradiol (Chart 4). Estradiol only partially reversed the effects seen with 5 μM tamoxifen, 2.5 and 5 μM DMT, and 2.5 μM 4OHT. The effect of 5 μM 4OHT on percentage of S-phase cells was unaffected by the presence of 500 nM 17β-estradiol.

These patterns of reversal by 17β-estradiol of the cell cycle kinetic changes induced by tamoxifen and its metabolites (Chart 4) appeared to parallel the changes in cell numbers seen in earlier experiments (Chart 2). To elucidate the nature of this correlation, percentage of S-phase cells was determined for cells treated with antiestrogens (0.01 to 5 μM) in the presence or absence of a 10-fold lower concentration of 17β-estradiol (1 to 500 nM). The population-doubling time was calculated and hence the doubling (proliferation) rate. Over the concentration range tested, the proliferation rate was found to correlate with the logarithm of percentage of S-phase cells, with a coefficient of determination, $r^2 = 0.96$ (Chart 5).

Binding of Tamoxifen Metabolites to ER. Direct saturation analysis data for the binding of tritiated tamoxifen, trans-4-hydroxytamoxifen, and 17β-estradiol to MCF-7 cell cytosol showed that each compound was bound to a single saturable
Chart 4. Effects of tamoxifen, DMT, and 4OHT, with or without 17β-estradiol, on the percentage of MCF-7 cells in S phase of the cell cycle. After 30 hr of exponential growth, cells were treated with tamoxifen, DMT, or 4OHT with or without a 10-fold lower dose of 17β-estradiol. Treated medium was changed daily. Cells were harvested at 144 hr and subjected to flow cytometry as described under "Materials and Methods." Columns, percentage of cells in S phase of the cell cycle, mean of duplicate flasks from 3 experiments (n = 6); bars, S.E.

Chart 5. Correlation between MCF-7 proliferation rate and the proportion of cells in S phase of the cell cycle following antiestrogen treatment. Cells were treated with tamoxifen, DMT, or 4OHT (0.01 to 5 μM) with or without a 10-fold lower dose of 17β-estradiol as described in Chart 4, and at 144 hr cells were harvested and counted, and the percentage of S-phase cells was calculated from DNA histograms obtained by flow cytometry. Points, mean of duplicate flasks from 3 experiments (n = 6) for cells treated with tamoxifen (●), tamoxifen and 17β-estradiol (▲), DMT (△), DMT and 17β-estradiol (○), 4OHT (◊), or 4OHT and 17β-estradiol (▲); coefficient of determination, r² = 0.96.

binding component, presumably the ER (Chart 6A). The apparent equilibrium dissociation constant, Kd, was lowest for estradiol, 0.10 nm, while trans-4-hydroxytamoxifen was bound with about one-half this affinity, i.e., Kd = 0.18 nm. Tamoxifen, by contrast, had comparatively weak affinity for the cytoplasmic ER, the Kd of 3.7 nm being almost 40-fold higher than that for 17β-estradiol.

These data were in good agreement with the competitive binding data illustrated in Chart 6B. Under these assay conditions, 4OHT had 41 ± 3% (n = 4) of the affinity of 17β-estradiol for the cytoplasmic ER while tamoxifen and DMT were equipotent with RBAs (17β-estradiol = 100%) of 2 ± 1%.

DISCUSSION

The data presented herein confirm and extend previous studies from this (27, 28, 32) and other laboratories (4, 6, 14, 19) on the effects of tamoxifen (4, 6, 14, 19, 27, 28, 32) and its metabolites (6) on the growth kinetics of human breast cancer cells in vitro. In agreement with the findings of others, these data illustrate that tamoxifen is a potent inhibitor of cell proliferation in vitro (4, 6, 14, 19), that many of its effects can be reversed by simultaneous administration of 17β-estradiol (6, 19), and that 4OHT, which has enhanced affinity for ER (2, 6, 16, 33), is a more potent growth inhibitor than tamoxifen in vitro (6). More importantly, this study reveals that DMT and 4OHT have both estrogen-reversible and estrogen-irreversible components to their inhibition of cell proliferation in vitro, with the former being highly correlated with affinity for ER, that both compounds demonstrate cytotoxicity at doses >5 μM but with different potencies, and that both compounds induce changes in cell cycle kinetics similar to those seen with tamoxifen, i.e., an increase in the proportion of G0-G1-phase cells at the expense of S- and G2-
M-phase cells (27, 28). In the estrogen-reversible range where cytotoxicity is not evident, the degree of change in cell cycle parameters was correlated with affinity for ER, and in addition the rate of cell proliferation for all 3 antiestrogens over a 50-fold concentration range, in the presence or absence of a 10-fold lower dose of 17β-estradiol, was highly correlated with the proportion of cells in S phase. This suggested that estrogen and antiestrogen were competing for a common pathway that determined the rate of entry into S phase and thus the rate of cell proliferation. Furthermore, DMT, the quantitatively most important metabolite in human plasma and tumor tissue (7), was shown to be at least equipotent with tamoxifen in affecting cell proliferation kinetics, which raises the possibility of a major role for this metabolite in tumor responses to tamoxifen in vivo. Many of these points require further expansion and discussion.

The present experimental design with daily medium changes differs from our previous study (28) where medium remained unchanged and drug unreplenished over a 6-day period. Interestingly, the sensitivity to tamoxifen was very similar in both studies, adding support to the conclusion that tamoxifen is not rapidly metabolized or inactivated in this system (14).

Data reported here for the affinity of 4OHT and tamoxifen for the cytoplasmic ER from MCF-7 cells are in good agreement with those reported by others (6, 8). However, the estimates of affinity for 17β-estradiol are somewhat higher than reported by others (4, 8), leading us to the conclusion that in MCF-7 cells 4OHT is bound somewhat less tightly than 17β-estradiol. This is in agreement with the competitive binding data presented in Chart 2C; we are unable to explain this apparent anomaly. However, the relative concentrations of 17β-estradiol required for complete reversal were 5- to 20-fold lower than would be predicted from the measured RBAs of 17β-estradiol and antiestrogen for ER. The explanation for this is unclear but could include the following. (a) Occupation of all ER sites may not be essential for maximal MCF-7 proliferation. Indeed, Lippman et al. (19) reported maximal induction of MCF-7 cell proliferation at concentrations of 17β-estradiol sufficient to saturate only about 10 to 15% of ER sites. (b) Incomplete solubility of the antiestrogens in culture medium (6) may account for some of the decrease in expected activity relative to 17β-estradiol. (c) Binding studies done in cell-free systems at lowered temperatures may not reflect the situation in whole cells where there could be differential permeability of cells to 17β-estradiol and antiestrogens or compartmentalization of antiestrogens within the cell, e.g., by binding to the high-affinity microsomal antiestrogen binding site (23, 30).

The involvement of ER in the cytotoxic effect, however, seems unlikely in view of its 17β-estradiol irreversibility and the lack of correlation between relative potency and affinity for ER. There has, however, been some interest recently in the possibility that tamoxifen may act through its high-affinity binding site since a tamoxifen-resistant MCF-7 line with similar ER concentration to the wild type had markedly decreased levels of antiestrogen binding site (17). In addition, a study from this laboratory showed a possible relationship between the cytotoxic properties of some clomiphene analogues and their affinity for this antiestrogen binding site (24). The present data do not, however, support such a relationship since the relative potency of these compounds in producing cytotoxicity did not correlate with their affinity for the antiestrogen binding site (tamoxifen > 4OHT > DMT). Assessment of the likely relative contributions of tamoxifen and its metabolites to its antitumor effects in vivo depends on which, if any, of the effects in vitro correlate with tumor regression in vivo. If the noncytotoxic antiproliferative effect is relevant, then consideration of both the relative levels of 4OHT, tamoxifen, and DMT (7) in human tumors (0.02:1:1.5) and their relative potencies (150:1:1) would indicate that all 3 compounds may be important, with relative contributions of 3:1:1.5, respectively. If the cytotoxic effect is more relevant, however, DMT is likely to

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be the major antitumor agent, with a lesser contribution from tamoxifen.

An understanding of the relative contributions of these metabolites to the action of tamoxifen is important for interpretation of tamoxifen pharmacokinetics in humans. During chronic administration, tamoxifen reaches steady-state levels after 4 weeks, in contrast to the 8 weeks required for DMT (25). This means that the maximal combined effects of tamoxifen and its metabolites may not be reached until much later than previously assumed, and this gives added weight to the proposal for tamoxifen "loading" schedules (9, 34).

In summary, these data show that the 2 metabolites, DMT and 4OHT, have similar actions to tamoxifen on MCF-7 cells in vitro in that they have 17β-estradiol-reversible and 17β-estradiol-irreversible components to their growth-inhibitory activity that are associated with accumulation of cells in the G0-G1 phase of the cell cycle. Regardless of which of these components may be relevant to the antitumor action of tamoxifen in vivo, consideration of the reported intratumor concentrations of the metabolites (7) together with their relative potency in producing growth inhibition in vitro leads to the conclusion that at least one of these metabolites is likely to make a major contribution to the clinical antitumor action of tamoxifen. Since the potency of these compounds in producing the 17β-estradiol-reversible growth inhibition is correlated with affinity for ER, it is likely that an ER-mediated mechanism is a major regulator of the distribution of cells between the previously described "slowly cycling" and "rapidly cycling" pools (28, 32) and thus the rate of cell proliferation. The 17β-estradiol-irreversible, cytotoxic component, however, seems unlikely to be ER mediated since no such correlation could be found between the potency of these compounds in producing this effect and their affinities for ER.

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