ABSTRACT

The triphenylethylene antiestrogen tamoxifen (TAM) is believed to exert its antitumor effect via the estrogen receptor (ER). To test this hypothesis and to differentiate between ER-mediated and general cytotoxic effects of TAM, the growth-inhibitory effects of TAM and its in vivo metabolite 4-hydroxytamoxifen (OH-TAM) have been studied in five continuous human cancer cell lines, MCF7 and T47D (mammary carcinoma, ER positive), BT20 and MDA-MB-231 (mammary carcinoma, ER negative), and ME8 (melanoma, ER negative). All five cell lines are completely killed by concentrations of TAM and OH-TAM above $10^{-6}$ M, regardless of ER status. TAM and OH-TAM have little effect on the ER-negative lines at concentrations below $10^{-6}$ M, whereas the ER-positive lines are highly sensitive to TAM at $10^{-7}$ M and to OH-TAM at $10^{-9}$ M. Inhibition of growth parallels the relative affinity of these drugs for the ER. We conclude that, above $10^{-6}$ M, the growth-inhibitory effects of TAM and OH-TAM in tissue culture are the results of a mechanism other than that via the ER system and that at lower concentrations are the true ER-mediated effects seen. Plasma concentrations of TAM and OH-TAM in breast cancer patients treated with TAM are in the same range as the concentrations in vivo at which growth inhibition is seen, leading to the conclusion that both compounds contribute to the overall effect of TAM in vivo.

INTRODUCTION

In recent years, the triphenylethylene antiestrogen TAM has been used successfully in the treatment of ER-positive breast cancer (23, 24), but the molecular basis of its antitumor activity is not well understood. It has been suggested that the mechanism resulting in the growth-inhibitory effects of TAM is mediated by the ER (6, 9, 10), especially as a metabolite of TAM, OH-TAM, which is formed in vivo in rats (12), chicks (3), and humans (1), is a more potent antiestrogen in vitro than TAM itself (6) and has a higher affinity for the ER (3, 11).

Several laboratories have reported that triphenylethylene antiestrogens also bind to other saturable binding sites present in several tissues, including human breast cancers (19, 29, 30). These binding sites are distinct from the ER in that they bind triphenylethylene antiestrogens but not steroidal or nonsteroidal estrogens. Recent work seems to suggest that these sites are probably not directly involved in the estrogen antagonism of antiestrogens, but that they may be important in affecting the distribution of antiestrogens within the cell (19).

The effect of TAM on the growth of primarily one ER-positive breast cancer cell line (MCF7) has been reported by several groups using concentrations of TAM in the range of 1 to 10 $\mu$M ($10^{-6}$ to $10^{-5}$ M) (6, 14, 18, 19). It has been shown that growth inhibition of MCF7 cells by TAM results in an accumulation of cells in the G0-G1 phase of the cell cycle (13, 22, 28, 31). Interestingly, however, the inhibition of growth of an ER-negative breast cancer cell line, BT-20, by the same concentrations of TAM ($10^{-6}$ to $10^{-5}$ M) is also accompanied by an accumulation of cells in G0-G1 (13). Since the BT-20 cell line does not contain ERs (16), the effect of TAM at these high concentrations must be via a non-receptor-mediated, nonspecific cytotoxic mechanism.

In order to distinguish between ER-mediated and nonspecific cytotoxic effects of TAM and OH-TAM, we have studied the growth response of several ER-positive and ER-negative cell lines to a range of concentrations of TAM, OH-TAM, and estradiol (10$^{-10}$ to $10^{-5}$ M). As there have been several reports of the serum concentration in culture media affecting the response of cultured cells to antiestrogens (5, 6), 3 different concentrations of serum, treated with charcoal to remove endogenous estrogens (18), were used in these growth studies. The relative affinities of TAM and OH-TAM for the ER have been measured by competition studies using whole cells growing in monolayer culture (32) and correlated with the growth-inhibitory effects of these 2 antiestrogens.

MATERIALS AND METHODS

Materials. 17$\beta$-[2,4,6,7-$^3$H]Estradiol (104 Ci/mmol) was obtained from New England Nuclear (Boston, MA). TAM and OH-TAM were supplied by I.C.I., Ltd. (Macclesfield, England).

Tissue culture media and reagents were obtained from Seromed (Seromed GmbH, Munich, Germany), and insulin, estradiol, diethylstilbestrol, and BSA were from Sigma Chemical Co. (St. Louis, MO). Culture dishes and plates were obtained from Falcon (Becton Dickinson and Co., Oxnard, CA), Lux (Lux Scientific Corp., Newbury Park, CA), or Costar (Cambridge, MA).

Cell Lines and Growth Conditions. Five human cell lines were used in the studies described below: T47D and MCF7 (human breast carcinoma, ER positive) (16); BT-20 and MDA-MB-231 (human breast carcinoma, ER negative) (16); and ME-8 (human melanoma, ER negative). T47D, MCF7, and MDA-MB-231 were supplied by the Human Cell Culture Bank, National Cancer Institute, Bethesda, MD; ME-8, by the Ludwig Institute for Cancer Research, Lausanne, Switzerland; and BT-20, by the American Type Culture Collection (Rockville, MD). T47D, MCF7, and ME-8 were cultured routinely in minimal essential medium supplemented with L-glutamine (2 mM), nonessential amino acids, gentamicin (5 mg/ml), insulin (200 ng/ml), and 10% (v/v) fetal calf serum. MCF7 cells were cultured in the same basic medium plus 10% calf serum, and BT-20, in minimal essential medium containing nonessential amino acids, gentamicin (2 mM), pyruvate (1 mM), gentamicin (5 mg/ml), and 10% fetal calf serum. Trypsin-EDTA in Hank's balanced salt solution was used for cell passages. Cells were maintained in a humidified atmosphere of 95% air-5% CO$_2$ at 37°C.

Received September 19, 1983; accepted December 27, 1983.
Cells were grown in T-75 flasks in the media described above. At the beginning of the experimental period, the cells were removed from the T-75 flasks with trypsin-EDTA solution and were plated in either 24-well (30,000 cells/ml/well) or 12-well (60,000 cells/2 ml/well) plates in medium plus 10% serum. Twenty-four hr later, this medium was removed and replaced with medium containing the appropriate concentration of charcoal-treated fetal calf serum (18) ( charcoal-treated calf serum for MCF7 cells) and the drug to be tested.

**Growth Studies.** Each cell line was plated as described above, and after 24 hr, the medium was removed and replaced with fresh medium containing 2, 5, or 10% charcoal-treated serum and estradiol, TAM, or OH-TAM (10^{-10} to 10^{-4} M). The hormones were stored in solution in ethanol at -20° and were added to the growth medium to give a final concentration of 0.1% ethanol. Control cultures were fed with medium containing 0.1% ethanol. Fresh medium plus drug was made when the medium was changed every 2 days. After 7 days of drug treatment, the cells were washed twice with cold 0.1 N perchloric acid, and the cells in the multwell plates were stored at −20° until required for DNA assay. DNA was measured by the method of Burton (4).

**Effect of Estradiol on Cell Growth after Treatment of Cells with TAM or OH-TAM.** T47D and MCF7 cells were grown in the presence of 10^{-10} M TAM or OH-TAM as described above. After 5 days of drug treatment, one-half of the drug-treated cells was treated with 10^{-14} M estradiol during 4 days, while the other half continued to be treated with 10^{-7} M TAM or OH-TAM. Cells were harvested, and DNA was measured on Days 1, 5, and 10 of the treatment period.

**Total ER Levels in Cells in Monolayer Culture.** ER levels were determined directly in the multwell plates (32). Cells were plated and grown in medium plus 2% charcoal-treated serum as described above. After 8 days of growth, medium was removed and replaced with 0.5 ml medium plus 0.1% BSA containing 12 to 0.375 nM [3H]estradiol ± 100-fold molar excess diethylstilbestrol to assess nonspecific binding. After 2-hr incubation at 37°, the medium was removed and replaced with 1.0 ml phosphate buffer (5 mM sodium phosphate-0.25 M sucrose-10% glycerol, pH 7.4) containing 0.5% BSA. After incubation at room temperature for 30 min, the cells were washed twice with 1.0 ml cold phosphate buffer (without BSA), and the radioactive steroid was extracted with 1.0 ml ethanol. The ethanol was transferred to a scintillation vial (Bio Vials; Beckman, Ltd.). Three ml of Beckman HB scintillator were added to each vial, and radioactivity was counted using a Beckman LS1800 scintillation counter. Specific binding was assessed by subtraction of the bound.

**RESULTS**

**ER Content of Cell Lines.** Table 1 shows the ER content of the 5 cell lines used in this work as determined by whole-cell uptake of radiolabeled estradiol (32). In agreement with other authors, only MCF7 and T47D are ER positive (18).

**Effects of Estradiol, TAM, and OH-TAM on the Growth of Cultured Cell Lines.** Charts 1 and 2 show the growth-inhibitory effects of TAM and OH-TAM on the 2 ER-positive breast cancer cell lines, T47D and MCF7, grown in media containing 2, 5, or 10% charcoal-treated serum. Growth of T47D cells seems to be little influenced by the serum content of the medium (Chart 1), whereas that of MCF7 is, growth being directly proportional to the medium serum content (Chart 2). It has been reported that T47D cells are unaffected by both estrogens and antiestrogens (15), but this does not seem to be the case in the experimental conditions we describe here. Estradiol (10^{-7} and 10^{-4} M) increases growth slightly, but above this concentration, the cytoxic effect, seen with all cell lines, is evident. TAM above 10^{-8} M causes a dose-dependent reduction in growth, whereas a marked growth reduction is seen already with 10^{-5} M OH-TAM. In all experiments, growth was assessed by measuring DNA, and we have found that DNA and cell number, as measured with a Coulter Counter, are related linearly (data not shown). In our hands, the growth of MCF7 cells is unaffected by estradiol (Chart 2), but there is a concentration-dependent decrease in cell growth with both TAM and OH-TAM and complete cell death at 10^{-5} M antiestrogen, as seen with T47D cells. The effect of the antiestrogens on MCF7 cells is dependent upon the serum content of the medium, growth inhibition being most marked in 2% serum.

Charts 3 and 4 show the effects of different concentrations of estradiol, TAM, and OH-TAM on the 2 ER-negative breast cancer cell lines, MB-231 and BT-20. Both cell lines are sensitive to the serum content of the medium, but up to a concentration of 10^{-6} M, neither estradiol nor the antiestrogens have any effect on cell growth. Above this concentration, a marked decrease in cell growth is seen with all compounds, the effects being more pronounced at lower serum concentrations.

The effects of estradiol, TAM, and OH-TAM on the human melanoma cell line ME-8 are shown in Chart 5. Again, there is little effect of any compound below 10^{-6} M, and above this, a marked cytotoxic effect is seen.

**Effect of Estrogen on Drug-treated Cells.** The so-called "rescue" of cells from the growth-inhibitory effects of TAM and OH-TAM by estrogen (6, 14) is shown in Chart 6. Treatment of both MCF7 and T47D cells with 10^{-7} M TAM or OH-TAM results in significant growth inhibition after 5 days. If these cells are then treated with 10^{-8} M estradiol, cell growth accelerates to the control rate. By the end of the experimental period, growth of cells treated with TAM has been completely restored, whereas that of OH-TAM-treated cells is only partially reversed. This suggests that growth inhibition and its reversal are ER mediated, as the efficiency of estradiol to reverse the inhibitory effect parallels the affinity of the antiestrogen for the ER as measured by competition of TAM and OH-TAM for the ER in whole cells (Chart 7). At concentrations of antiestrogen above 10^{-6} M, the effect of TAM and OH-TAM in all cell lines is nonreversible by estradiol, suggesting a nonspecific non-ER-mediated cytotoxic mechanism.
DISCUSSION

The growth-inhibitory effects of TAM and OH-TAM on the 5 cell lines described above can clearly be separated into 2 distinct components: (a) an effect which occurs in all cell lines irrespective of ER status at concentrations of drug of 1 \( \mu M \) and above; and (b) an effect at concentrations of drug between 1 nm and 1 \( \mu M \) which is confined to cells which are ER positive. At high concentrations (above 1 \( \mu M \)), estradiol itself is also cytotoxic to all cell lines (Charts 1 to 5).

As shown by other workers (5, 6), the concentration of serum in the medium used for cell culture affects the growth of cells and their response to estrogens or antiestrogens (Charts 1 to 5). The pattern of growth inhibition described above is, however, irrespective of the serum content of the medium, so at all concentrations, we see cytotoxicity above 1 \( \mu M \) and a possible ER-mediated effect in ER-positive cells below 1 \( \mu M \) drug.

It therefore appears that the response of ER-positive tumors to TAM can be explained in terms of a receptor-mediated event. Plasma levels of TAM and OH-TAM in postmenopausal breast
cancer patients treated with TAM have been reported to be between 100 and 400 ng/ml (2.5 × 10^{-7} to 10^{-6} M) for TAM and 2 and 12 ng/ml (5 × 10^{-9} and 3 × 10^{-8} M) for OH-TAM (7, 8, 11). Actual tissue levels of TAM and OH-TAM may be higher (7) than this, as the antiestrogen binding protein may help to concentrate the antiestrogen within the cell (19), although at the moment, little is known about the function of this antiestrogen binding. The cell lines used in our studies all have the antiestrogen binding protein, and all take up large amounts of radiolabeled TAM and OH-TAM,3 so the intracellular concentrations of TAM and OH-TAM may well be higher than that in the surrounding culture medium. The reported plasma levels of TAM and OH-TAM correlate well with the culture medium concentration of drug at which we see growth-inhibitory effects of TAM and OH-TAM on ER-positive MCF7 and T47D cells.

Plasma concentrations of TAM in postmenopausal breast cancer patients treated with TAM are approximately 3 to 4 orders of magnitude higher than plasma estradiol levels (7). The affinity of TAM for ER is somewhere between 1,000- and 10,000-fold lower than that of estradiol and also that of OH-TAM, of which the affinity for the ER is closer to that of estradiol itself (Chart 7). Thus, the plasma concentrations of TAM and OH-TAM are consistent with the model for the action of the drug in which the antiestrogen acts on the tumor tissue by association with ER proteins. The fact that both TAM and OH-TAM are present in plasma at concentrations which could lead to antiestrogenic effects implies that both compounds probably contribute to the overall effect. Another metabolite of TAM, N-desmethyltamoxifen, which is present in plasma at concentrations higher than that of TAM itself (7) but which has a lower affinity for ER than does TAM, probably also plays a role (11, 25). It has been shown that, when conversion of TAM to OH-TAM is blocked by substitution at position 4 by fluoro, chloro, or methyl groups, estrogen antagonism still occurs, but it is less than when TAM, which can be hydroxylated at position 4, is administered (2, 17). Thus, hydroxylation of TAM is probably not required for antiestrogenic activity, but it can enhance the effect.

The phenomenon of rescue by estradiol of antiestrogen-treated cells, as well as supporting the idea that the mechanism of action is ER mediated, emphasizes the point that patients undergoing treatment with antiestrogens will respond less well when they have high estrogen levels. The ER is blocked by TAM at high concentrations (10^{-6} M; Chart 7) and by OH-TAM at lower concentrations, paralleling the growth-inhibitory effects of antiestrogens. A similar situation has been shown clinically as ER levels in patients' tumors are in general higher pre-TAM treatment than...
posttherapy (20, 21, 33). The presence of high estrogen levels, such as the premenopausal patient, would tend to rescue treated cells by displacing the antiestrogen from ER and thereby reduce the response of the patient to the treatment.

At higher concentration (above 1 μM), TAM and OH-TAM are cytotoxic to all cell lines (Charts 1 to 5), this effect not being reversed by estradiol. The cytotoxic effect is complete at 10 μM and is independent of the ER status of the cell. We suggest that care should be taken in interpreting results already published, when investigations into the mechanism of action of antiestrogens have been made using concentrations of the drugs in vitro systems of 1 to 10 μM or even higher (13, 28, 31). It appears from our results described above that such high concentrations of antiestrogens are very cytotoxic, and any effect seen as a result of such levels of drug may well be nonspecific and unrelated to any actual estrogen antagonist action. At similar high concentrations (1 to 10 μM), estradiol itself inhibits the incorporation of tritiated thymidine into DNA and eventually kills both ER-positive and ER-negative human breast cancer cell lines (18). Riley et al. (26) have suggested that high-dose estradiol has nonspecific effects on the cell membrane and cytoskeleton structure and that this may be responsible for the inhibition of cell growth caused by high-level estradiol in vitro. Such a nonspecific effect may also occur with high levels of hydrophobic compounds, such as TAM and OH-TAM, resulting in their cytotoxicity.

Our results confirm those already reported by Coezy et al. (6) and Lippman et al. (18), both of whom have shown inhibition of growth of MCF7 cells with 0.1 μM TAM and rescue by estradiol. Here, we show similar effects with another ER-positive human breast cancer cell line (T47D), a cell line which has been said previously to be refractive to both estrogens and antiestrogens, and contrast these effects with those occurring in ER-negative cell lines. From these studies, we conclude that the antiestrogen TAM, which is currently used in the treatment of ER-positive breast cancer patients, exerts its antitumor effect via the ER and that its metabolite, OH-TAM, also plays an important role in the in vivo situation.

ACKNOWLEDGMENTS

We thank C. Wiedmer and U. Gygli for preparation of the manuscript.

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Estrogen Receptor-mediated and Cytotoxic Effects of the Antiestrogens Tamoxifen and 4-Hydroxytamoxifen

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