Bioactivities, Estrogen Receptor Interactions, and Plasminogen Activator-inducing Activities of Tamoxifen and Hydroxytamoxifen Isomers in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Tamoxifen is used widely in the treatment of endocrine-responsive breast cancers in humans. Studies were undertaken to examine the biological character (estrogenic-antiestrogenic properties) and estrogen receptor (ER) interaction of the cis- and trans-isomers of tamoxifen and hydroxytamoxifen in MCF-7 human breast cancer cells. For each compound, the following parameters were monitored: affinity for ER and effects on cellular ER levels; stimulation-inhibition of cell growth, plasminogen activator activity, and cellular progesterone receptor levels; and isomer interconversion and metabolism in vitro. The relative binding affinities of the compounds cis-tamoxifen, trans-tamoxifen, cis-hydroxytamoxifen, and trans-hydroxytamoxifen for cytosol ER were 0.3, 2.5, 1.8, and 310%, respectively, in which the affinity of estradiol is considered 100%. cis-Tamoxifen behaved as a weak estrogen agonist in all assays, while trans-tamoxifen was an effective estrogen antagonist. cis-Tamoxifen behaved like estradiol in stimulating MCF-7 cell growth and increasing plasminogen activator activity and cellular progesterone receptor content, although very much higher concentrations of cis-tamoxifen (10^-8 M) were needed to achieve the levels of stimulation observed with 10^-10 M estradiol. trans-Tamoxifen and trans-hydroxytamoxifen suppressed cell growth, inhibited plasminogen activator activity of control cells, and suppressed estradiol-stimulation of plasminogen activator activity, and they evoked minimal increases in cellular progesterone receptor levels. trans-Hydroxytamoxifen had a 100-fold increased affinity for ER and was approximately 100-times more potent than was trans-tamoxifen in suppressing cell growth and plasminogen activator activity. cis-Hydroxytamoxifen behaved as an estrogen antagonist, suppressing cell growth and plasminogen activator activity, and it elicited minimal increases in progesterone receptor levels. This apparently paradoxical behavior of cis-hydroxytamoxifen was shown to be due to the fact that the cis- and trans-hydroxytamoxifen readily undergo isomeric interconversion upon exposure to our cell culture conditions, resulting in substantial accumulation of the higher-affinity trans-hydroxytamoxifen in the nuclear ER fraction of cells. In contrast to the facile interconversion of the hydroxytamoxifen isomers, there is no metabolism or interconversion of the parent compounds cis- and trans-tamoxifen in vitro.

Hence, by the criteria we have used, the biological characters of trans-tamoxifen and trans-hydroxytamoxifen are similar, the major difference being the approximately 100-fold enhanced potency of the hydroxylated form. In contrast, cis-tamoxifen is an estrogen with a biopotency roughly proportional to its ER binding affinity. The apparent estrogen-antagonistic character of cis-hydroxytamoxifen appears attributable to isomerization to trans-hydroxytamoxifen in vitro. With all tamoxifen isomers, there was a good correlation between suppression or stimulation of plasminogen activator activity and suppression or stimulation of cell growth, suggesting that cell-associated plasminogen activator activity may serve as a good marker for estrogen action in breast cancer cells.

INTRODUCTION

The antiestrogen tamoxifen is a triphenylethylene compound that is used widely in the treatment of human breast cancer (1, 15, 18, 37). Interestingly, the geometric isomers of some antiestrogens have been shown to differ markedly in their agonistic or antagonistic character (14, 36). Studies in rat uterus have shown that the trans-isomer of tamoxifen, the form used in human breast cancer treatment, is a potent antiestrogen, while the cis-geometric isomer is a pure estrogen with no estrogen-antagonistic actions (20, 33). Metabolic studies have shown that one of the routes of tamoxifen metabolism in vivo involves conversion to a hydroxylated form, trans-hydroxytamoxifen (1-[4-(2-dimethylaminoethoxy)phenyl]-1-[4-hydroxyphenyl]-2-phenylbut-1(Z)-ene), that has a higher affinity for estrogen receptor and is an even more potent estrogen antagonist than is trans-tamoxifen (1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(Z)-ene, ICI 46,474) (1, 2, 7, 9, 12, 13, 19). Few studies, however, have examined the biological character of the cis and trans-isomers of tamoxifen, and their hydroxylated forms, cis-hydroxytamoxifen (1-[4-(2-dimethylaminoethoxy)phenyl]-1-[4-hydroxyphenyl]-2-phenyl-but-1(E)-ene) and trans-hydroxytamoxifen, in human breast cancer cells. Hence, our aim in this study was to determine the antiestrogenic/estrogenic character and biopotencies of the cis- and trans-isomers of tamoxifen and the hydroxylated form of these compounds in estrogen receptor-rich human breast cancer cells.

For these studies, we have used the MCF-7 human breast cancer cell line that has been used frequently as a model for estrogen-responsive human breast cancer, and we have studied cell growth, plasminogen activator activity, and cellular progesterone receptor levels as indices of estrogenic-antiestrogenic character. Our studies show that, as in the rat uterus, the cis-isomer of tamoxifen is a pure, although weak, estrogen, while the trans-isomer of tamoxifen is an effective estrogen antagonist. Compared with trans-tamoxifen, trans-hydroxytamoxifen has an

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approximately 100-fold increased affinity for the estrogen receptor in MCF-7 cells and a markedly enhanced antiestrogenic potency. Surprisingly, however, the hydroxylated form of cis-tamoxifen (1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1-(E)-ene), ICI 47,899, cis-hydroxytamoxifen, behaves as a quite potent antiestrogen. This apparent paradoxical behavior is shown to be due to the fact that cis-hydroxytamoxifen is converted rapidly to trans-hydroxytamoxifen under the conditions of cell culture. Our findings indicate that the stereochemistry of these triphenylethylenes influences markedly their biological character in human breast cancer cells. In addition, stimulation of plasminogen activator production is a very sensitive end point of estrogen agonist action in MCF-7 breast cancer cells.

MATERIALS AND METHODS

Chemicals and Materials. The nonradioactive compounds cis-tamoxifen, trans-tamoxifen, trans-4-hydroxytamoxifen, and cis-4-hydroxytamoxifen were kindly provided by ICI, Ltd., (Macclesfield, U. K.), or Stuart Pharmaceuticals (Wilmington, Del.). The pure trans-isomer of [1H]tamoxifen (12.2 Ci/mmol), the pure cis-isomer of [3H]tamoxifen (6.5 Ci/mmol), trans-[3H]hydroxytamoxifen (22 Ci/mmol, 99% pure), and cis-[3H]hydroxytamoxifen (29 Ci/mmol, 99% pure) were synthesized by Drs. D. Robertson and J. Katzenellenbogen, University of Illinois, Urbana, Ill. (32). The purity of all unlabeled and tritiated tamoxifen and hydroxytamoxifen isomers appeared to be >99% pure by these chromatographic criteria.

[2,4,6,7-3H]Estradiol (108 Ci/mmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). The synthetic progesterin [6,7,8,9-3H]R5020 (17.21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione) (69 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). All media, sera, and antibiotics used to culture the MCF-7 cells were obtained from Grand Island Biological Co. (Grand Island, N. Y.). Insulin, hydrocortisone, and estradiol were purchased from Sigma Chemical Co. (St. Louis, Mo.). HEPES was purchased from Grand Island Biological Co. BZIPAR was synthesized and purified according to the procedure of Leytus et al. (24). Fluorescein monop-guanidinoacetate was synthesized and purified according to the procedure of Melhado et al. (26). Plasminogen was purified from fresh dog plasma as described in Leytus et al. (23) and was stored at −20° in HEPES-buffered saline (0.01 M HEPES, pH 7.2-0.137 M NaCl-2.68 mM KCl-0.91 mM CaCl2-0.49 mM MgCl2). Human urokinase was purchased from Henley & Co., New York, N. Y.

The tolulene-based scintillation fluid was 0.50% PPO and 0.03% POPP in toluene. The Triton:xylene-based scintillant was 0.3% 2,5-diphenyloxazole:0.02% POPP:25% Triton X-114 in xylene.

Cell Culture. MCF-7 human breast cancer cells were originally obtained by Dr. Charles McGrath of the Michigan Cancer Foundation (Detroit, Mich.) and were grown in plastic T-150 flasks in MEM supplemented with 10% HEPES buffer, gentamicin (50 μg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), bovine insulin (6 ng/ml), hydrocortisone (3.75 ng/ml), and 5% calf serum that had been treated with dextran-coated charcoal for 45 min at 55° to remove endogenous hormones (10). These cells were used for all studies reported in this manuscript except for one series of cell growth experiments (Chart 4) which utilized MCF-7 cells obtained from Dr. M. E. Lippman (NIH, Bethesda, Md.). These cells were grown in plastic T-150 flasks in IMEM (Grand Island Biological Co.) containing 10% HEPES buffer, gentamicin (50 μg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), hydrocortisone (3.75 ng/ml), insulin (0.02 unit/ml), and 5% fetal calf serum.

Cell Growth Experiments. To determine the effect of tamoxifen isomers on cell growth, MCF-7 cells from the Michigan Cancer Founda-

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of progesterin binding, suggesting no contribution from glucocorticoid receptor. After incubation, samples were assayed for bound [3H]R5020 using hydroxylapatite as described for the estrogen receptor assay above.

**Competitive Binding Assays.** Cells were harvested from 150-sq cm Corning T-flasks, and cytosol was prepared at a protein concentration of approximately 2 mg/ml in phosphate buffer. Aliquots of this 180,000 x g supernatant were then incubated with various concentrations of radioinert estradiol, cis- or trans-tamoxifen, or cis- or trans-hydroxytamoxifen, and 2.5 x 10^-8 M [3H]estradiol for 16 hr at 0-4° and were then analyzed exactly as described previously (21). The relative binding ability of each competitor is taken as the ratio of the concentration of radioinert estradiol/competitor required to inhibit one-half of the specific [3H]estradiol binding, with the affinity of estradiol set at 100%.

**Assay for Plasminogen Activator Activity.** The plasminogen activator activity of the cells was measured by a 2-step assay using the rhodamine-based compound BZIPAR as a substrate for plasmin (24). The substrate is nonfluorescent, but cleavage by plasmin of an amide bond between arginine and rhodamine yields a product, mono(carbocenzoxyisoleuc-cyprolylgly)rhodamine, that is highly fluorescent. The K_m for dog plasmin is 4.25 sec^-1 and the K_M is 34.1 μM (30). The 2-step assay consisted of incubating cells with dog plasminogen for an appropriate time interval. The amount of plasmin formed was then measured by incubating an aliquot of the reaction mixture with BZIPAR and determining the rate of increase in fluorescence. To convert from relative fluorescence units per min to a molar concentration of plasmin, a standard curve was required. This was obtained by incubating aliquots from a stock solution of plasmin, the concentration of which was determined by an active site titration with fluorescein monoo-p-quinidinobenzoate (26), with BZIPAR and measuring the increase in fluorescence after 15 min. The slope of this line was used to convert relative fluorescence units per min to a molar concentration of plasmin (30).

In our assays, 25 to 200 x 10^5 cells were seeded into microwells (Costar, 96-well tissue culture cluster plates) and incubated in a sealed humidified jar at 37° for 24 hr. Plasminogen [50 μl of dog plasminogen (1 mg/ml)] was then layered over the attached cells, that had been washed twice with HEPES-buffered saline to remove traces of media and serum, and the cells were incubated with plasminogen at 37° for 2 hr. To measure the amount of plasmin formed, a 0.04-ml aliquot was removed and added to a 0.96-ml solution of 50 μM BZIPAR in 0.01 M HEPES, pH 7.2, containing 15% (v/v) ethanol and 5% (v/v) dimethyformamide at 25°. After an appropriate time interval (15 min), the increase in fluorescence was measured in a Fluoroscan microplate photometer (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 492 nm and 523 nm, respectively. The fluorescence was standardized several times each day using a polymethacrylate block embedded with rhodamine B to ensure that the relative fluorescence was comparable in different experiments. The experiments were performed (data not shown) that indicated the rate of activation of plasminogen during the 2 hr incubation of plasminogen with cells was constant and therefore proportional to the amount of plasminogen activator. Conversion of relative fluorescence units per min to molar concentrations of plasmin was accomplished by using a standard curve as detailed previously (30).

**Thin-Layer Chromatography and High-Performance Liquid Chromatography of Tamoxifen and Hydroxytamoxifen Isomers.** Thin-layer chromatography was done on plastic-backed silica gel plates (SILICA GEL 60 F254, Merck No. 5775), as described previously (33) using 2 solvent systems, benzene:triethylamine (9:1, v/v) for optimum resolution of the tamoxifen isomers and benzene:piperidine (9:1, v/v) for optimum resolution of the hydroxytamoxifen isomers. Standard reference compounds (33) were applied to each chromatogram along with the radiolabeled compound or cell media extract; the relative mobility of the standards was measured under UV.

In studies aimed at examining the possible interconversions of cis- or trans-tamoxifen and cis- or trans-hydroxytamoxifen, MCF-7 cells were incubated for times up to 72 hr with 3 x 10^-7 M titrated compound in media containing serum exactly as done for usual experiments; or media:serum alone (without cells) was incubated with 3 x 10^-8 M tritiated compound. The media were then collected and extracted 3 times with 10 volumes of ethyl acetate. The cells were harvested and homogenized, and the cell homogenate was extracted 3 times with 10 volumes of ethyl acetate. Unlabeled carrier compounds were added to the extracts, and the extracts were dried under nitrogen gas, redissolved in ethanol, and analyzed by thin-layer chromatography in the 2 solvent systems according to the methods described above. Portions of the media and cell fractions were also directly analyzed by high-performance liquid chromatography without prior organic solvent extraction of the radioactive.

High-performance liquid chromatographic analysis of hydroxytamoxifen isomers was performed on a Spectro-Physics 8600 system using a Beckman-Altex Model 153 with an Isco UA-4 UV detector set at 280 nm. Separations were performed on a 25-cm x 4.6-mm 5-μm C8 silica gel reversed-phase column (IBD) with a guard column (5 cm x 3 mm) packed with Co-Pel Pak (Whatman). The mobile phase was saturated with silica by passage of the solvent through a precolumn packed with (0.05 to 0.2 mm) silica gel upstream from the injector (Brinkman).

Two solvent systems were used to separate the hydroxytamoxifen isomers: System A: acetonitrile (65%):0.25 M diethylammonium phosphate, pH 7.5 (35%) (elution time of trans-hydroxytamoxifen, 13 min, and of cis-hydroxytamoxifen, 19 min, at 0.8 ml per min); and System B: methanol (73%):0.25 M diethylammonium phosphate, pH 9.0 (27%) (elution time of trans-hydroxytamoxifen, 25 min, and of cis-hydroxytamoxifen, 31 min). In each case, carrier quantities of both cis- and trans-hydroxytamoxifen (approximately 2 μg) were added to the sample of medium or to the cell fraction immediately after the fraction was obtained. The samples were either analyzed directly (up to 200 μl), or they were extracted twice with 5 volumes of ethyl acetate, and the extracts were dried and evaporated under a gentle stream of nitrogen. The addition of carrier hydroxytamoxifens and the avoidance of exposure of evaporated samples to air was important to avoid the formation of undesired degradation products.

**RESULTS**

**Binding Affinity of the Tamoxifen and Hydroxytamoxifen Isomers for the MCF-7 Estrogen Receptor.** The binding affinity of the tamoxifen and hydroxytamoxifen isomers for the cytosol MCF-7 estrogen receptor was determined by competitive binding analysis. Comparison of the concentration of these compounds needed to produce a 50% decrease in the specific binding of tritiated estradiol (Chart 1) indicates that trans-tamoxifen has an affinity 2.5% that of estradiol; cis-tamoxifen has an affinity 0.3% that of estradiol. Trans-hydroxytamoxifen, known to be a metabolite of tamoxifen, has an affinity 310% that of estradiol, while cis-hydroxytamoxifen has an affinity of only 1.8%. These relative binding affinities are similar to those obtained previously for binding to the estrogen receptor in rat uterine cytosol (33). These results demonstrate the dramatic increase in receptor affinity that occurs upon hydroxylation of the tamoxifen isomers, and they indicate that the trans-isomers have a much higher receptor binding affinity than do the corresponding cis-isomers, with this geometric dependence being even greater for the hydroxytamoxifen isomers.

**Effects of cis- and trans-Tamoxifen and Hydroxytamoxifen Isomers on Cytosol Receptor Levels in MCF-7 Cells.** Estrogen and antiestrogen are known to interact with the estrogen receptor system of target cells, evoking the depletion of cytosol receptor sites and the movement of these sites to the nucleus. In studies depicted in Chart 2, we have examined the dose relationships for this depletion of cytosol receptor sites by tamoxifen and hydroxytamoxifen isomers. MCF-7 cells were incu-
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Ox (\textsuperscript{73}OH-Tam) (33)

Chart 1. Competitive binding assay of tamoxifen and hydroxytamoxifen isomers. MCF-7 cytosol was incubated for 16 hr at 0-4\degree C with the stated concentration of competitor (10\textsuperscript{-10} to 10\textsuperscript{-5} M) and 2.5 \times 10\textsuperscript{-8} M \([\text{H}]\)estradiol (E\textsubscript{2}). After incubation, charcoal-dextran (15% by volume, 15 min at 0-4\degree C) was added to adsorb unbound ligand, and radioactivity was determined in the supernatant. Numbers in parentheses, relative binding affinity of each compound for receptor (estradiol = 100). cis-Tam, c/s-tamoxifen; cis-OH-Tam, c/s-hydroxytamoxifen; trans-OH-Tam, trans-hydroxytamoxifen; trans-Tam, trans-tamoxifen.

Chart 2. Depletion of cytosol estrogen receptor sites following a 5-day treatment with various concentrations of tamoxifen or hydroxytamoxifen isomers. Near-confluent flasks of cells were incubated for 5 days with the indicated compounds, with the medium changed daily, so that fresh medium containing hormone was added daily. The cells were then harvested and assayed for cytosol estrogen receptor as described in "Materials and Methods." Each point represents data obtained in duplicate from one near-confluent T-150 flask of cells. Values obtained in 2 additional experiments did not vary by more than 10%. cis-Tam, c/s-tamoxifen; cis-OH-Tam, c/s-hydroxytamoxifen; trans-OH-Tam, trans-hydroxytamoxifen; trans-Tam, trans-tamoxifen.

Chart 3. Effect of the c/s- and trans-isomers of tamoxifen and hydroxytamoxifen on the growth of MCF-7 cells. Cells were grown in T-25 flasks in the presence of the indicated concentration of tamoxifen or hydroxytamoxifen isomer or 10\textsuperscript{-8} M estradiol (E\textsubscript{2}) and media with hormones were changed every other day. At several time points during the 10-day growth period, triplicate flasks of cells were counted. The control cell doubling time was 2.2 days. Data are expressed as the percentage of the control cell number measured after 10 days of growth. Bars, S.E. cis-Tam, c/s-tamoxifen; trans-Tam, trans-tamoxifen; cis-OH-Tam, c/s-hydroxytamoxifen; trans-OH-Tam, trans-hydroxytamoxifen.

Effects of Tamoxifen and Hydroxytamoxifen Isomers on MCF-7 Cell Growth. Under our conditions of assay of our MCF-7 cells, estradiol does not stimulate cell growth, as reported also by others (4, 5, 29), but trans-tamoxifen and trans-hydroxytamoxifen are effective inhibitors of cell growth, with trans-hydroxytamoxifen being 100 times as potent as its nonhydroxylated form, consistent with their different receptor affinities (Chart 3). cis-Tamoxifen, like estradiol, did not stimulate or suppress growth of these cells but, surprisingly, cis-hydroxytamoxifen appeared to be a potent inhibitor of cell growth, being approximately 10-fold more potent than was trans-tamoxifen in this regard.

However, when we assayed for the effects of the tamoxifen isomers in other MCF-7 cells, obtained from Dr. M. Lippman, which are reported to show estradiol-stimulated growth (27), we observed that both estradiol and cis-tamoxifen evoked a markedly enhanced cell growth (Chart 4). cis-Tamoxifen was a much less potent agonist, half-maximal stimulation of growth requiring approximately 10\textsuperscript{-7} M cis-tamoxifen but only approximately 10\textsuperscript{-11} M estradiol. trans-Tamoxifen, trans-hydroxytamoxifen, and cis-hydroxytamoxifen suppressed the growth of these MCF-7 cells, as seen in Chart 3 (data not presented).
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**Effects of Tamoxifen and Hydroxytamoxifen Isomers on Stimulation of Plasminogen Activator Activity.** Plasminogen activator activity is stimulated markedly by low concentrations of estradiol in MCF-7 cells. As seen in Chart 5, this stimulation, which is monitored by a change in fluorescence, is proportional to cell number. Maximal stimulation, approximately 3- to 4-fold, was achieved with 10^{-9} M estradiol, and marked stimulation was seen with 10^{-10} M estradiol (Chart 6). Time course studies (not shown) indicated optimal stimulation of plasminogen activator activity by estradiol at 1 to 8 days (i.e., optimal stimulation occurred on Day 1 and remained stable through Day 8) and by cis-tamoxifen at 1 to 4 days of hormone stimulation; stimulation by cis-tamoxifen was substantially reduced after 6 or 8 days of hormone stimulation, for reasons that we do not yet understand. Hence, all studies were done using a 4-day exposure to hormone.

cis-Tamoxifen behaved as an estrogen, although a weak one in terms of this response, in that it stimulated plasminogen activator activity at high concentrations. However, even at the highest concentration tested (3 x 10^{-6} M), stimulation was only approximately 75% of that achieved with estradiol. trans-Tamoxifen and trans-hydroxytamoxifen, as well as cis-hydroxytamoxifen, markedly suppressed plasminogen activator activity below the control level. In addition, trans-tamoxifen, trans-hydroxytamoxifen, and cis-hydroxytamoxifen (at 10^{-8} to 10^{-6} M) suppressed to very low levels (10 to 25%) the stimulation of plasminogen activator activity produced by 10^{-9} M estradiol or 10^{-10} M cis-tamoxifen (data not presented). Hence, these 3 tamoxifen compounds appeared to be effective antagonists of this response.

**Effects of Tamoxifen and Hydroxytamoxifen Isomers on Stimulation of Cellular Progesterone Receptor Content.** Increases in progesterone receptor content have been used as an index of estrogen action in MCF-7 cells (10, 16, 17). In studies on the time course of progesterone receptor stimulation by the different compounds (data not shown), we found similar, maximal increases at 3, 4, 5, or 6 days of exposure to estradiol or cis-tamoxifen. Hence, a 5-day time point was selected for comparative evaluation of progesterone receptor stimulation by the tamoxifens. Chart 7 summarizes data on the effect of each compound on progesterone receptor stimulation in MCF-7 cells. Estradiol, as anticipated, was a potent stimulator of progesterone receptor; optimal (600%) increases in progesterone receptor levels were observed at 10^{-10} M estradiol, and marked stimulation was maintained at doses as high as 10^{-6} M. cis-Tamoxifen evoked increases in progesterone receptor levels as great as those evoked by estradiol, but much higher (10^{-8} M) concentrations of cis-tamoxifen were needed to achieve this 600% of control stimulation. trans-Hydroxytamoxifen and cis-hydroxyta-
cells (Table 1). The isomerization was time dependent, reaching 25% conversion to trans-hydroxytamoxifen in the media by 2 days. Because of its higher affinity for the estrogen receptor, the trans-hydroxytamoxifen was accumulated preferentially in the nuclear receptor fraction of the cells, accounting for approximately 85% of the total radioactivity of the nuclear salt extract at 48 hr. In a parallel study (Table 1), trans-hydroxytamoxifen was shown to undergo isomerization to cis-hydroxytamoxifen to an equivalent extent, although there was very little accumulation of cis-hydroxytamoxifen in the nuclear receptor fraction, presumably because of its relatively low affinity for the estrogen receptor.

**DISCUSSION**

These studies in MCF-7 human breast cancer cells indicate that the biological character of the cis- and trans-isomers of tamoxifen differ. cis-Tamoxifen has a low affinity for the estrogen receptor but appears to be a pure, albeit weak, estrogen in these cells. Full stimulation of progesterone receptor was achieved...
with this compound, and marked stimulation of plasminogen activator activity was also evoked by cis-tamoxifen. In addition, cis-tamoxifen behaved like estradiol in terms of its effect on the growth of MCF-7 cells. In MCF-7 cells under growth conditions in which estradiol markedly stimulated cell proliferation, cis-tamoxifen evoked nearly equivalent cell growth stimulation, but very much higher concentrations of cis-tamoxifen than estradiol were required. Hence, this compound acted much like estradiol in MCF-7 cells in terms of these 3 biological parameters.

trans-Tamoxifen, in contrast, suppressed the growth of MCF-7 cells, inhibited plasminogen activator activity of control cells, and suppressed estrogen-stimulation of plasminogen activator activity, and it evoked minimal increases in progesterone receptor levels in MCF-7 cells. The hydroxylated form of tamoxifen, which is a metabolite of tamoxifen in vivo (1, 2, 8, 12, 13, 33), had a 100-fold increased affinity for estrogen receptor (9) and was approximately 100 times more potent in suppressing cell growth and plasminogen activator activity, and it was also very ineffective in evoking progesterone receptor increases. Hence, by the criteria we have used, the biological character of trans-tamoxifen and trans-hydroxytamoxifen are similar, the major difference being the enhanced potency of the hydroxylated form.

A similar correlation between estrogen receptor affinity and inhibition of cell growth was reported for trans-tamoxifen and trans-hydroxytamoxifen by Coezy et al. (7).

In contrast to this situation, seen with trans-tamoxifen and trans-hydroxytamoxifen, the apparent biological character of cis-hydroxytamoxifen was very different from that of cis-tamoxifen. While cis-tamoxifen appeared to be an estrogen, cis-hydroxytamoxifen behaved as an estrogen antagonist. It suppressed cell growth and the plasminogen activator activity of control and estradiol-stimulated cells, and it elicited submaximal increases in progesterone receptor levels, appearing much like trans-hydroxytamoxifen and trans-tamoxifen in these assays. This apparently paradoxical behavior of cis-hydroxytamoxifen is due to the fact that the cis- and trans-hydroxytamoxifen isomerize readily upon exposure to our culture conditions. Interestingly, this isomeric interconversion is not attributable to metabolism by the cells but, rather, occurs in the media alone at 37°C (Table 1). In the case of cis-hydroxytamoxifen incubations, trans-hydroxytamoxifen is generated in sufficient amounts to likely account for the apparent antiestrogenic activity of cis-hydroxytamoxifen. In the case of trans-hydroxytamoxifen incubations, substantial amounts of cis-hydroxytamoxifen are also generated but, due to the much higher receptor affinity of trans-hydroxytamoxifen, almost exclusively trans-hydroxytamoxifen accumulated in the nuclear receptor (Table 1), and only an antiestrogenic activity is observed. The antiestrogenic character of cis-hydroxytamoxifen, reported recently in rat pituitary tumor cells in vitro (25), might also be explained by a similar conversion to trans-hydroxytamoxifen.

Of note is the relative ease of isomerization of the hydroxytamoxifen as compared with the parent compounds, where no isomer interconversion was detected here in MCF-7 cells, nor in studies in rat uterus (33). There is precedent for the facile geometric isomerization of p-hydroxyx stilbene systems such as is present in the hydroxytamoxifen (32). The interconversion of the hydroxytamoxifen was temperature dependent. Little, if any, isomerization of cis-hydroxytamoxifen occurred at 4°C, suggesting that the relative binding affinity of cis-hydroxytamoxifen measured by competitive binding assay at 4°C is probably an accurate reflection of its estrogen receptor affinity. The approximatively 5- to 10-fold enhanced potency of cis-hydroxytamoxifen in cell assays at 37°C, compared to its relative binding affinity, is consistent with our observation of isomerization to the more potent trans-hydroxytamoxifen form. Studies are in progress to identify the factors responsible for the isomeric interconversion of the hydroxytamoxifens and, ultimately, if possible, to attempt to prevent isomerization so that the true biological character of cis-hydroxytamoxifen can be determined.

Trans-hydroxytamoxifen and N-desmethy tamoxifen are the major metabolites of tamoxifen found in breast cancer patients receiving tamoxifen (1, 9, 13). Whether some of the trans-hydroxytamoxifen is normally isomerized to cis-hydroxytamoxifen in vivo is not known. However, in related studies, Jordan et al. (20) have reported that cis-hydroxytamoxifen is an antiestrogen in uterine weight gain studies in rats in vivo, and they have speculated that, in vivo, isomerization of the hydroxytamoxifens might occur. While it is tempting to speculate, on the basis of our cell culture results, that isomeric interconversion of the hydroxytamoxifens might be occurring in vivo, until the factors that are promoting the isomerization in cell culture are understood, it would be premature to extrapolate our findings to the in vivo situation.

Finally, it is of interest that plasminogen activator activity serves as a useful marker of estrogen agonist activity and is able to distinguish estrogens from antiestrogens (4—6). Plasminogen activator activity is stimulated by low, physiological concentrations of estradiol, and antiestrogens such as trans-tamoxifen and trans-hydroxytamoxifen do not increase plasminogen activator activity in MCF-7 cells. In contrast, cis-tamoxifen is able to increase markedly plasminogen activator activity of MCF-7 cells. That plasminogen activator activity is a preferable marker for distinguishing estrogens from antiestrogens is seen from comparing the effects of antiestrogens on progesterone receptor and plasminogen activator activity. While antiestrogens such as trans-tamoxifen do not increase plasminogen activator activity (5) and actually suppress the control cell level of plasminogen activator activity, trans-hydroxytamoxifen and other antiestrogens (10) increase cellular progesterone receptor levels, making progesterone receptor a less good marker of estrogenic/antiestrogenic character. The good correlation between growth suppression and suppression of plasminogen activator activity in these breast cancer cells by different tamoxifen suggests that plasminogen activator activity serves as a good correlate of growth in these cells. Plasminogen activator activity is frequently under hormonal control and has been shown to be increased in uterus (22, 30) and mammary gland (28) during periods of marked tissue growth and remodeling. The correlation between plasminogen activator activity, estrogen sensitivity, as measured by estrogen receptor levels, and metastatic potential of human breast cancers remains under active investigation (34, 35).

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