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FACILE GEOMETRIC ISOMERIZATION OF PHENOLIC NON-STEROIDAL ESTROGENS AND ANTIESTROGENS: LIMITATIONS TO THE INTERPRETATION OF EXPERIMENTS CHARACTERIZING THE ACTIVITY OF INDIVIDUAL ISOMERS

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Summary—In many estrogen responsive systems the isomers of tamoxifen are known to have different biological character–the *trans* isomer is generally an antagonist and the *cis* isomer an agonist. Attempts to similarly characterize the isomers of hydroxytamoxifen (which differ greatly in their affinity for the estrogen receptor) are shown to be complicated by their facile isomerization. This isomerization was studied in cultures of estrogen receptor positive MCF-7 human breast cancer cells and monitored by HPLC under reversed phase conditions. Hydroxytamoxifen isomers that are initially 99% pure, undergo a time and temperature dependent isomerization, so that after 2 days in tissue culture medium at 37°C they have isomerized to the extent of 20%. This isomerization occurs in the cell-free medium alone and cannot be attributed to a metabolic conversion by the cells. The isomerization occurs much more slowly at 4 than at 37°C and can be reduced considerably by various antioxidants (butylated hydroxytoluene, ascorbate, α-tocopherol, retinoic acid and retinal); however, at concentrations that block isomerization, these antioxidants are toxic to the cells. Although the medium contains both the *cis* and *trans* isomers of hydroxytamoxifen, the MCF-7 cells preferentially accumulate the *trans* isomer and the material associated with the nuclear estrogen receptor is, in all cases, mainly the higher affinity *trans* isomer. A similar preference of the estrogen receptor for the *trans* isomer is seen with diethylstilbestrol, resulting again in almost exclusive accumulation of the *trans* isomer in the receptor binding site.

These experiments indicate the importance of verifying the isomer compositions of easily isomerizable non-steroidal estrogens and antiestrogens, such as diethylstilbestrol and hydroxytamoxifen, both in stock solutions and in experimental samples (especially those derived from receptor-associated material), so as to ascertain that the activity of the individual isomers is being correctly assigned.

INTRODUCTION

There has been a great deal of interest in the development of non-steroidal compounds having estrogenic and antiestrogenic activity as agents for regulating fertility and controlling the growth of estrogen-dependent neoplasms [1–3]. Many studies have been concerned with the fact that those compounds that embody a stilbene system may display very different activities, depending upon the geometry of the central carbon–carbon double bond; not only is the potency of these two isomers different, but their agonist–antagonist activity may differ as well [4–9].

Perhaps the most striking difference is displayed by the isomers of tamoxifen: the Z isomer, termed *trans*-tamoxifen or Nolvadex (ICI-46,474), is in most systems an estrogen antagonist of considerable potency, while the E isomer (*cis*-tamoxifen, ICI-47, 699) is very weak, and as far as has been characterized, is exclusively an agonist [4–9]. The isomers of some other non-steroidal antiestrogens have also been shown to have different activity, although this appears not always to be the case [8]. The *cis* and *trans* isomers of the non-steroidal estrogen diethylstilbestrol are also reported to have different bioactivities [10, 11].

Obviously, the validity of both *in vivo* and *in vitro* experiments that are designed to determine the potency and character of the individual isomers of these non-steroidal estrogens and antiestrogens depends upon the maintenance of the isomeric integrity of these compounds during the assay period. For example, if during the assay, an isomer of low potency is undergoing conversion to the corresponding high potency isomer, the results of the assay would be compromised. In general, isomerization during bioassay is not a problem for those non-steroidal compounds lacking a 4- or 4′-hydroxyl group on the stilbene system. However, with those compounds having such a function, either present in the molecule or generated during the assay by metabolism, isomerization may well be occurring.

In this paper, we describe studies that document the isomerization of hydroxytamoxifen in experiments performed in cell culture and reveal the capacity of the estrogen receptor to bind preferentially to the higher affinity isomer. We also demonstrate

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similar binding selectivity for the isomers of diethylstilbestrol. We analyze the isomerization of the hydroxytamoxifens and show that it is time and temperature dependent and that it can be reduced markedly by antioxidants. However, the levels of these agents needed to block isomerization totally are not tolerated by the cells. Therefore, it has not been possible to make an unambiguous determination of the activity of the low potency isomer, *cis*-hydroxytamoxifen, under these cell culture conditions.

EXPERIMENTAL

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, DE). The pure *trans* isomer of [^{3}H]tamoxifen (12.2 Ci/mmol), the pure *cis* isomer of [^{3}H]tamoxifen (6.5 Ci/mmol), [^{3}H]*trans*-hydroxytamoxifen (27 Ci/mmol, 99% pure) and [^{3}H]*cis*-hydroxytamoxifen (29 Ci/mmol, 99% pure) were synthesized by Drs D. Robertson and J. Katzenellenbogen as described [12]. [^{3}H]Diethylstilbestrol (81 Ci/mmol) was obtained from Amersham (Chicago, IL).

All media, sera, and antibiotics used to culture the MCF-7 cells were obtained from GIBCO (Grand Island, NY). Insulin, hydrocortisone, estradiol and antioxidants were purchased from Sigma Chemical Co. (St Louis, MO). *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES) was from GIBCO.

The Triton-xylene-based scintillant was 0.3%, 2,5-diphenyoxazole, 0.02% *p-bis*-[2-(5-phenyloxazoyl)]-benzene and 25% Triton X-114 in xylene.

Cell culture

MCF-7 human breast cancer cells were originally obtained from Dr Charles McGrath of the Michigan Cancer Foundation (Detroit, MI) and were grown in plastic T-150 flasks in Eagles Minimal Essential medium (MEM) supplemented with 10 mM HEPES buffer, gentamicin (50 μg/ml), penicillin (100 U/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°C to remove endogenous hormones [7].

Incubation of hydroxytamoxifen isomers in MCF-7 cells and preparation of cell fractions for chromatographic analysis

For examination of the interconversion of *cis*- or *trans*-hydroxytamoxifen, MCF-7 cells were incubated in T-150 flasks for times up to 48 h with 4×10^{-8} M tritiated compound in MEM media containing 5% charcoal dextran-treated serum, or media-serum alone (without cells) was incubated with the same concentration of tritiated compound. The media were then collected.

Cells were removed by a 10–20 min incubation at 37°C with 1.0 mM EDTA prepared in Ca^{+2}/Mg^{+2}-free Hank's Balanced Salt Solution (HBSS–EDTA), washed once at 4°C with 10 ml of Hank's Balanced Salt Solution (HBSS) and once in phosphate buffer (5 mM sodium phosphate, pH 7.4, 10 mM thioglycerol, and 10% glycerol). They were resuspended in 0.5 ml of phosphate buffer and homogenized in a Dounce homogenizer (Kontes) using the B-pestle until more than 95% of the cells were disrupted as determined by phase microscopy (ca 40 strokes). The homogenate was centrifuged 10 min at 800 *g* and the supernatant collected. The crude nuclear pellet was washed twice more with 0.5 ml phosphate buffer and the supernatants combined. The supernatant fraction (cytosol) was prepared by centrifugation at 180,000 *g* for 30 min at 4°C.

The crude nuclear pellet was resuspended in 0.375 ml of phosphate buffer to which 1.125 ml of TEK buffer (10 mM Tris–HCl (pH 8.5 at 4°C), 1.5 mM EDTA, 10 mM thioglycerol, 10% glycerol, and 0.8 M KCl) was added, and incubated for 1 h at 4°C with resuspension every 15 min. The suspension was centrifuged at 180,000 *g* (30 min), and the supernatant taken as the nuclear salt extract fraction.

In all cases, carrier quantities of both *cis*- and *trans*-hydroxytamoxifen (ca. 2 μg) were added to the sample of medium or to the cell fraction immediately after the fraction was obtained. The addition of carrier hydroxytamoxifens and the avoidance of exposure of evaporated samples to air were important to avoid the formation of undesired degradation products, as discussed further in Results.

Analysis of hydroxytamoxifen isomers by HPLC

Samples containing carrier amounts of unlabeled *cis*- and *trans*-hydroxytamoxifen were analyzed directly by reversed phase high performance liquid chromatography (without organic solvent extraction of the radioactivity). The chromatographic analysis was performed using a Spectro-Physics 8700 system with a Beckman-Altex Model 153 ultraviolet detector set at 280 nm; separations were performed on a 250 × 4.6 mm 5 μm C-8 silica gel reversed phase column (IBM or Supelco DB) with a guard column (50 × 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 (0.05–0.2 mm) silica gel (Brinkman) upstream from the injector.

Two solvent systems were used. System A, used to prepare the pure isomeric samples used in this study, was acetonitrile (65%)–0.25 M diethylammonium phosphate, pH 7.5 (35%)–elution time of *trans*-hydroxytamoxifen, 13 min and *cis*-hydroxytamoxifen, 19 min, at 0.8 ml per min. System B, used to separate mixtures of isomers and impurities, was methanol (73%)–0.25 M diethyl-

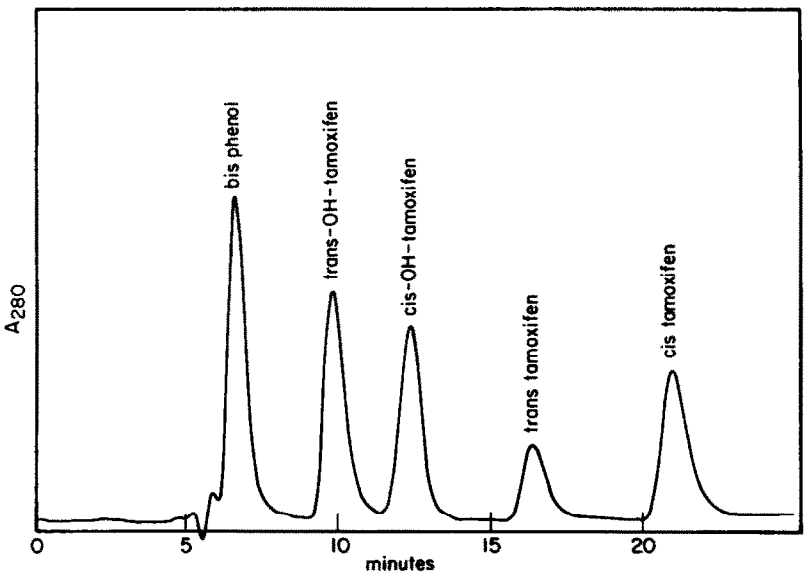

Fig. 1. HPLC separation of the *cis* and *trans* isomers of tamoxifen and hydroxytamoxifen, and a *bis*-phenol (1,1-*bis*(4-hydroxyphenyl)-2-phenyl-1-butene). A sample containing 2 μg each of the non-radiolabeled standards was injected onto a C-8 reversed phase column (Supelco DB-8 column 25 × 0.46 cm) and eluted with 82% methanol–18% 0.25 M diethylammonium phosphate (pH 9.0) at 0.8 ml/min. Absorbance was monitored at 280 nm with 0.05 absorbance units full scale.

ammonium phosphate, pH 9.0 (27%)–elution time of *trans*-hydroxytamoxifen, 22 min, and of *cis*-hydroxytamoxifen, 28 min at 0.8 ml per min.

RESULTS

Hydroxytamoxifen isomerization

Activity of hydroxytamoxifens in MCF-7 cells. The MCF-7 cell line, derived from a human breast tumor pleural effusion, has been used to characterize the agonist and antagonist activity of estrogens and antiestrogens, since antiestrogens such as *trans*-tamoxifen, suppress cell growth, while estrogens such as *cis*-tamoxifen, are stimulatory [7].

In a recent study, we attempted to use the MCF-7 cell system to study the agonist and antagonist properties of the isomers of hydroxytamoxifen [7]. Two interesting observations were made in these studies: First, while in competitive binding studies, *cis*-hydroxytamoxifen binds to the estrogen receptor only 0.6% as well as *trans*-hydroxytamoxifen, its potency in affecting cell growth rate was roughly 10% that of the *trans* isomer; second, the *cis* hydroxytamoxifen isomer, like the *trans*, was an antagonist of cell growth. This suggested that the less potent, possibly inherently agonistic *cis*-hydroxytamoxifen isomer was undergoing isomerization to the more potent and antagonistic *trans*-hydroxytamoxifen isomer. In order to investigate this process, we developed chromatographic separation methods that enabled us to assay the isomeric form of the hydroxytamoxifens present in the cell cultures and in different fractions of the cells at various times during the assay period.

HPLC analysis of hydroxytamoxifen isomers. Both isomers of tamoxifen and hydroxytamoxifen can be separated very cleanly by reversed phase HPLC on a deactivated C-8 column (IBM or Supelco DB Column). An example of such a separation, optimized for the isomers of tamoxifen, is shown in Fig. 1. The chromatogram in Fig. 2 shows the separation optimized for the more polar hydroxytamoxifen isomers; the *trans* and *cis* isomers elute at 22 and 28 min respectively, under these conditions.

In addition, the chromatogram in Fig. 2 shows the elution position of two impurities that originate from the hydroxytamoxifen isomers during manipulation and storage of small scale samples. The impurity

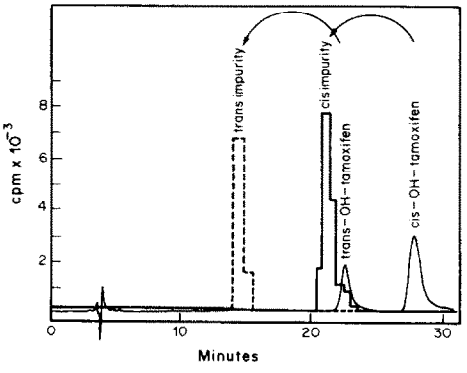

Fig. 2. HPLC separation of *cis* and *trans* hydroxytamoxifens and the impurities generated during the manipulation of small samples. The hydroxytamoxifen isomers and the degradation products were injected onto the column described in the legend to Fig. 1 and were eluted with 73% methanol–27% 0.25 M diethylammonium phosphate (pH 9.0) at 0.8 ml/min.

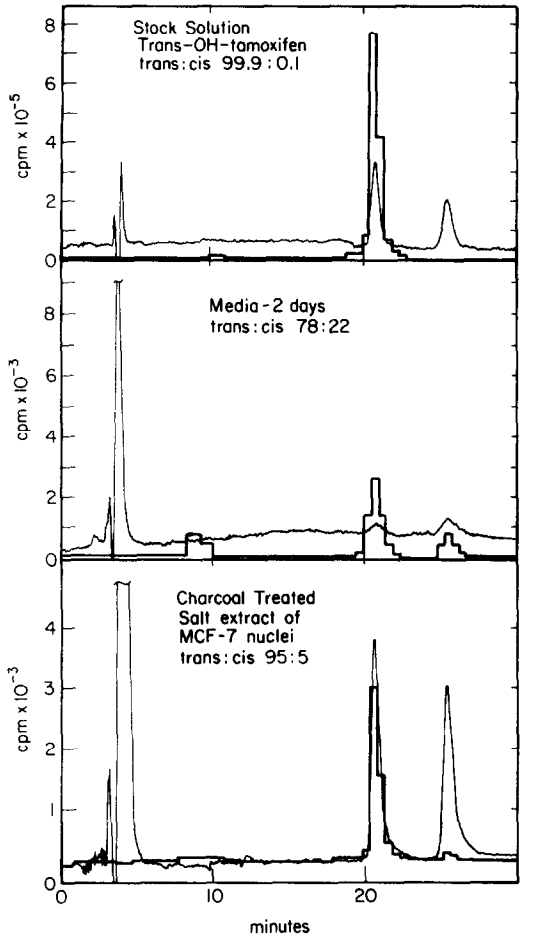

Fig. 3. HPLC analysis of isomerization of *trans*-hydroxytamoxifen to *cis*-hydroxytamoxifen. Freshly purified [^{3}H]*trans*-hydroxytamoxifen (Panel A) was added to MCF-7 cells in MEM medium containing 5% charcoal–dextran treated calf serum. After 2 days at 37°C, the composition of the media (Panel B) and the charcoal-treated salt extract of the cell nuclei (Panel C) were analyzed by HPLC after the addition of carrier *cis*- and *trans*-hydroxytamoxifen.

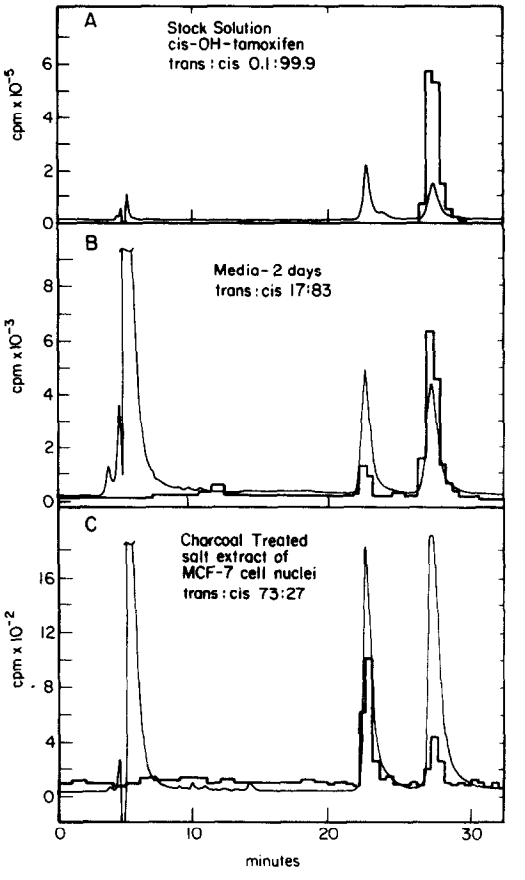

Fig. 4. HPLC analysis of the isomerization of *cis*-hydroxytamoxifen to *trans*-hydroxytamoxifen. Freshly-purified [^{3}H]*cis*-hydroxytamoxifen (Panel A) was placed in cell culture and analyzed, exactly as described in the legend to Fig. 3. Panel B shows the HPLC profile for material present in the media after 2 days; Panel C, the material from the charcoal-treated salt extract of the cell nuclei.

derived from *trans*-hydroxytamoxifen is well separated from both hydroxytamoxifen isomers, but that derived from *cis*-hydroxytamoxifen elutes just ahead of *trans*-hydroxytamoxifen; although the peaks of the hydroxytamoxifens are sharper in an acctonitrile–water system, this critical separation is cleaner in the methanol–water system used in this study.

While the identity of these impurities is not known, they seem to result from oxidation, and they may well be the cyclized phenanthrenes, as these have been reported elsewhere to form during the isolation of hydroxytamoxifen from biological samples [13]. Nevertheless, by careful addition of carrier quantities of the hydroxytamoxifens and by avoiding oxidizing conditions (careful solvent evaporation under nitrogen), the extent to which these impurities are produced can be reduced to very low levels.

Isomerization of hydroxytamoxifens in cell culture: isomer forms in the media and in cell fractions

When a culture of MCF-7 cells is incubated with a freshly-purified sample of [^{3}H]*trans*-hydroxytamoxifen (99.9% trans–0.1% *cis*, cf. Fig. 3A) for 2 days and then analyzed by HPLC after the addition of carrier *trans*- and *cis*-hydroxytamoxifen, the chromatogram of the media (Fig. 3B) shows that 22% of the total radioactivity present now is [^{3}H]*cis*-hydroxytamoxifen. HPLC analysis of various cell fractions (cf. Table 1 and Fig. 3C) shows a predominance of the *trans* isomer. In particular, analysis of the charcoal-stripped, salt-extracted nuclear fraction, that should contain almost exclusively material associated with the estrogen receptor, shows 95.4% *trans* isomer (Fig. 3C).

When the same experiment is performed with freshly-prepared [^{3}H]*cis*-hydroxytamoxifen (cf. Fig. 4 and Table 1), analysis of the media after 2 days indicated that the *cis* isomer has isomerized to *trans* to the extent of 17%. More striking, however, is the fact that the higher affinity *trans* isomer is preferen-

Table 1. Isomerization of hydroxytamoxifen

Sample	*trans*-OH-Tam		*cis*-OH-Tam	
	trans (%)	*cis* (%)	*trans* (%)	*cis* (%)
Initial	99.9	0.1	0.1	99.9
2 Day media	78.0	22.0	17.0	83.0
Cytosol	74.8	25.2	6.9	93.1
Salt-extracted nuclear	87.8	12.2	24.5	75.5
Charcoal-stripped salt-extracted nuclear	94.8	5.2	73.4	26.6

MCF-7 cells were incubated in Corning T-150 flasks with 4×10^{-8} M [^{3}H]*trans*-hydroxytamoxifen or 4×10^{-8} M[^{3}H]*cis*-hydroxytamoxifen in MEM tissue culture media containing 5% charcoal–dextran treated calf serum for 48 h at 37°C. After 48 h, the media was collected; samples of unlabeled *cis*- and *trans*-hydroxytamoxifen were added and the media were analyzed directly by HPLC. The cells were harvested and homogenized, and cell cytosol and nuclear salt extracts were prepared. Cell fractions, to which unlabeled standards were added, were also analyzed directly by HPLC.

tially accumulated by the receptor-containing fractions of the cell: The salt-extracted nuclear fraction consists of 24.5% *trans*–75.5% *cis* and the charcoal-stripped, salt-extracted nuclear fraction, 73.4% *trans*–26.6% *cis*. Thus, although the cells were originally treated with a sample of hydroxytamoxifen that contained only 0.1% of the *trans* isomer, after 2 days in culture, the nuclear estrogen receptor is occupied by an isomer composition that is nearly $\frac{3}{4}$ the *trans* isomer.

Factors affecting the rate of hydroxytamoxifen isomerization in cell culture

The progress of the isomerization (*trans*-hydroxytamoxifen to *cis*-hydroxytamoxifen) is shown in Fig. 5. The rate is essentially the same whether cells were present or absent from the culture media; the isomerization was reduced, however, if the incubation was placed under a nitrogen atmosphere, and reduced greatly when the temperature was maintained at 4°C. Neither procedure is compatible with cell viability.

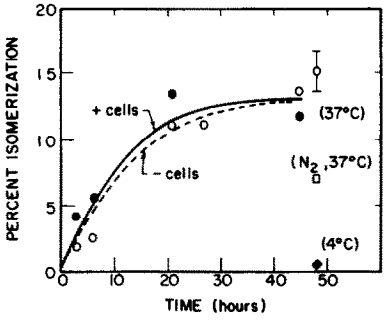

Fig. 5. Rate of isomerization of hydroxytamoxifen. Samples of [^{3}H]*trans*-hydroxytamoxifen containing, initially, less than 1% of the *cis* isomer, were placed in culture media both with (solid circles, solid line) and without (open circles, dashed line) MCF-7 cells at 37°C. At the times indicated, portions of the media were removed and analyzed by HPLC for the isomeric composition of the hydroxytamoxifen. The open circle shown at 48 h indicates the average extent of isomerization (± standard error of the mean) from four different experiments noted after 48 h at 37°C without cells; the point labeled N_2 is the extent of isomerization noted after 48 h (at 37°C without cells) under a nitrogen atmosphere and that labeled 4°C, the isomerization after 48 h at 4°C (without cells).

Since the isomerization of hydroxytamoxifen appears to be promoted under oxidative conditions, we attempted to retard the rate of isomerization by inclusion of known antioxidants and radical scavengers, such as butylated hydroxytoluene (BHT), ascorbate, α-tocopherol, retinoic acid, retinal and dimethylsulfoxide (DMSO). The results are shown in Fig. 6.

After 2 days in culture media, a sample of *trans*-hydroxytamoxifen that was initially only 1% *cis* had isomerized to the extent of 16% *cis*. Each antioxidant (except DMSO) was capable of reducing the extent of isomerization, often to a considerable degree, but only at high concentrations (e.g. ascorbate and α-tocopherol at 10^{-3} M, BHT at 10^{-4} M; at 4.5×10^{-4} M BHT, isomerization was only 1.6%). Since the viability of MCF-7 cells is reduced markedly by concentrations of these antioxidants even 10-fold lower than those needed to suppress the isomerization of the hydroxytamoxifens (data not presented), these antioxidants did not prove useful in suppressing the isomerization during cell culture experiments.

While stilbene systems are known to undergo *cis–trans* isomerization when irradiated with u.v. light [14], the hydroxytamoxifen samples in these experiments were incubated in the dark and were manipulated in plastic or glassware that absorbs all light of wavelength shorter than 350 or 300 nm. In addition, since high concentrations of the antioxidants (e.g. 4.5×10^{-4} M BHT) reduce isomerization nearly completely, this too suggests that photoisomerization is not taking place to any appreciable extent.

Estrogen receptor binding selectivity for the isomers of diethylstilbestrol

The isomers of diethylstilbestrol are known to undergo *cis–trans* isomerization readily [10, 11]. Although the isomerization may be catalyzed by radicals, as is the case of the hydroxytamoxifens, it also can precede in an interesting bimolecular fashion [11].

In the experiment presented in Fig. 7, the capacity of the estrogen receptor from rat uterine cytosol to bind selectively to the *trans* isomer of diethylstilbestrol is demonstrated. The same HPLC system used to analyze the isomers of hydroxytamoxifen was able to separate the isomers of diethylstilbestrol (Fig. 7A). When a sample of [^{3}H]diethylstilbestrol that contained an equilibrium mixture of isomers (78% *trans*–22% *cis*) was allowed to bind to rat uterine cytosol (Fig. 7B) and the free ligand then stripped with charcoal–dextran, the total bound material was found to be 97% *trans* (Fig. 7C, Table 2). In a parallel experiment, the same concentration of [^{3}H]diethylstilbestrol was added together with an

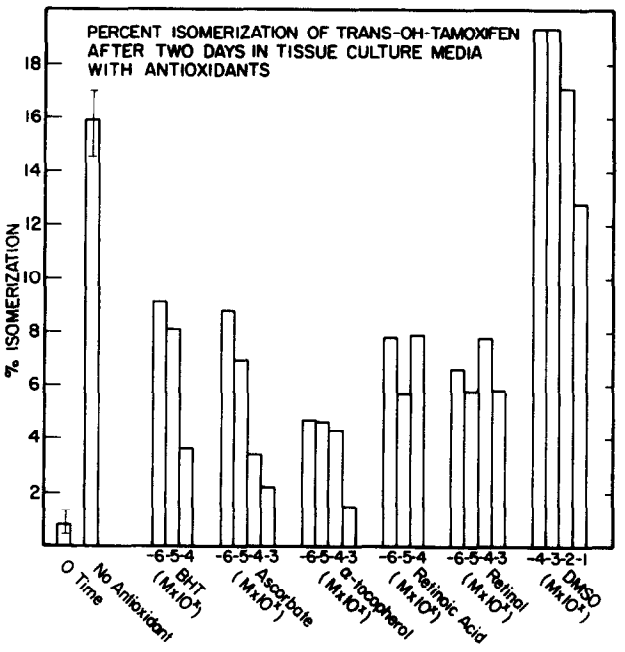

Fig. 6. Effects of antioxidants on the extent of hydroxytamoxifen isomerization. A sample of [^{3}H]*trans*-hydroxytamoxifen that contained less than 1% *cis* isomer was placed in culture media without cells together with the indicated concentrations of the antioxidants shown and incubated for 48 h at 37°C, at which time the isomer composition was assayed by HPLC. The extent of isomerization in the absence of antioxidant is shown as the average (± standard error of the mean) from 5 separate experiments. The other results are the average from two separate experiments.

excess of unlabeled estradiol to determine the isomer composition of the non-specifically bound material (Table 2). By subtraction of the isomeric composition found in these two determinations, it was possible to estimate that the diethylstilbestrol associated with the estrogen-specific binding sites is 99% *trans*.

DISCUSSION

When administered *in vivo*, tamoxifen is known to undergo extensive metabolism [15–17]. One of the metabolites, *trans*-hydroxytamoxifen, is bound by the estrogen receptor with much higher affinity than tamoxifen itself [6, 7, 9, 18]. It is thought that hydroxytamoxifen may be responsible for many of the antagonistic effects of tamoxifen administered *in vivo*. Therefore, it is important to determine the biological effectiveness of hydroxytamoxifen and its isomers.

Since they are generally free from the active metabolizing processes encountered *in vivo*, cell culture systems are often used to characterize the biological activity of metabolically labile compounds. MCF-7 and pituitary cell culture systems have been utilized to study the activity of isomers of tamoxifen and hydroxytamoxifen [7, 9, 19]. As expected, *trans*-tamoxifen was an antagonist and *cis*-tamoxifen an agonist; however, while *trans*-hydroxytamoxifen was an antagonist, *cis*-hydroxytamoxifen was also an antagonist. The *cis* isomer was also more potent in the cell culture assay than expected from its binding affinity for the estrogen receptor.

Under the conditions utilized in the MCF-7 cell culture study, however, we found that the isomers of hydroxytamoxifen underwent extensive interconversion, so that regardless of the fact that pure, single isomers were present at the start of the experiments, isomer mixtures were found 2 days later. This was especially true for the material found associated with the estrogen receptor, where, regardless of which isomer was used, the higher affinity *trans*-hydroxytamoxifen predominated after 2 days. Although we have shown here that antioxidants can block nearly completely the isomerization of hydroxytamoxifen, we were not able to suppress this isomerization completely under conditions consistent with maintenance of high cell viability; thus, we have concluded that it is not possible to establish the agonist/antagonist character of *cis*-hydroxytamoxifen in the absence of metabolism in this *in vitro* assay system.

In the study performed with diethylstilbestrol, we noted again that despite the isomer composition offered to a cell-free receptor preparation, the composition of the receptor-bound material strongly reflects the binding preference of the receptor, in this case for the *trans* isomer.

We have not done studies of the activity and isomeric stability of the hydroxytamoxifens *in vivo*: therefore, we cannot state to what degree isomerization may prove to be a problem in characterizing the activity of these isomers in whole animal studies. However, it is possible that because the environment

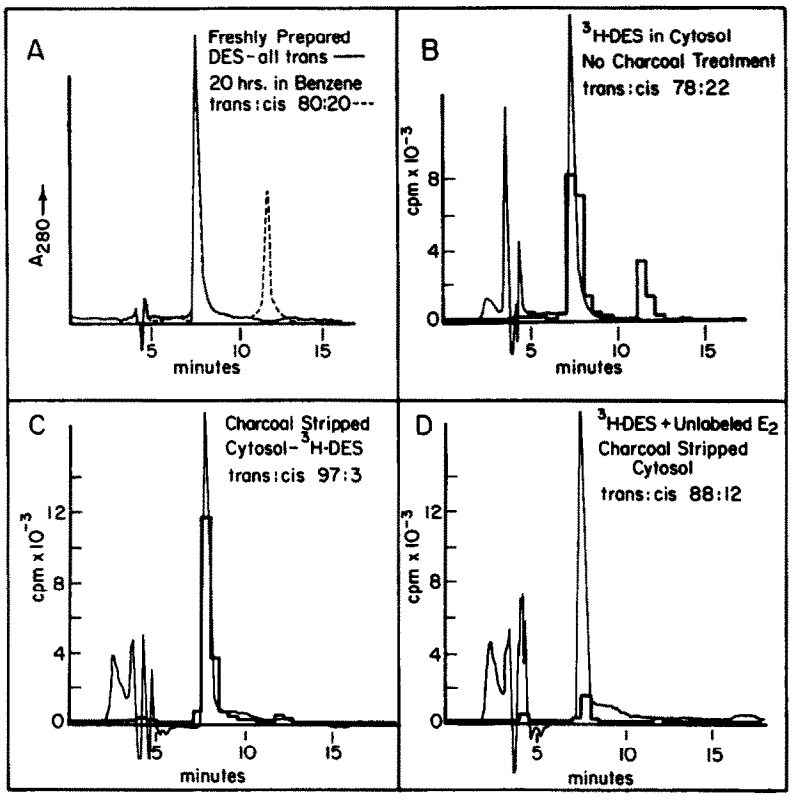

Fig. 7. Specificity of binding of the isomers of diethylstilbestrol by estrogen receptor. A sample of diethylstilbestrol, containing 80%—*trans* and 20%—*cis* isomers (Panel A), was incubated at a concentration of 31 nM with rat uterine cytosol at 4°C for 1 h. The isomer composition found in cytosol (Panel B), found associated with receptor after treatment with charcoal–dextran (Panel C), and found in a parallel incubation performed in the presence of 3000 nM of unlabeled estradiol (to measure non-specific binding, Panel D) was determined by HPLC under the same conditions used to analyze the isomers of hydroxytamoxifen (legend to Fig. 2).

in vivo may be more strictly reducing, that isomerization will be less of a problem. In any case, the result of our studies highlight the necessity of carrying out, in parallel with biological studies of the activity of readily isomerizable compounds, determinations of the isomeric integrity of the compound being tested and especially the isomeric composition of the receptor-associated material.

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Table 2. Stereospecificity of diethylstilbestrol (DES) binding by estrogen receptor

	Conditions	cpm (%) *trans*-DES	cpm (%) *cis*-DES
1.	[^{3}H]DES	17,184 (78%)	4761 (22%)
2.	[^{3}H]DES + CD	16,975 (97%)	442 (3%)
3.	[^{3}H]DES + E_2 + CD	1835 (88%)	247 (12%)
2–3	[^{3}H]DES receptor bound	15,140 (99%)	195 (1%)

[^{3}H]Diethylstilbestrol containing 80%—*trans* and 20%—*cis* isomers, was incubated at 31 nM with rat uterine cytosol at 4°C for 1 h. HPLC was used to determine the isomer composition in the cytosol (cf. Fig. 7) before (entry 1) and after treatment with charcoal–dextran (entry 2). A parallel incubation for non-specific binding determination (in the presence of 3000 nM of unlabeled estradiol) was similarly analyzed (entry 3). The composition of the receptor bound material was calculated from the difference between entries 2 and 3.

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