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Effects of *cis*- and *trans*-Tamoxifen Isomers on RNA Incorporation of Human Breast Cancer Cells

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SUMMARY

Cultured T47-D human breast cancer cells were used to investigate growth-inhibiting effects of the antiestrogen, *trans*-tamoxifen, on [^{3}H]Cyd incorporation into specific classes of nuclear and cytoplasmic RNA. The steroid agonist, 17β-estradiol, and the inactive *cis* isomer of tamoxifen were used as treatment controls to compare antiestrogen-induced changes in RNA metabolism, independent of estrogen receptor-binding properties. Using a 24-hr labeling interval, *trans*-tamoxifen produced a 1.4- to 4-fold enhanced incorporation into pre-rRNA species (20S, 32–45S), with slight reduction in mature 18S rRNA incorporation, and a 2- to 3-fold increased incorporation into 5S and 5.8S rRNA and 4–4.5S tRNA. Most notable, *trans*-tamoxifen enhanced incorporation into the less abundant low molecular weight U_1, U_3, and 7S RNA species by 3- to 6-fold. These findings were associated with an apparent reduction in pre-rRNA content and little change in U_1, U_3, or 7S RNA levels in antiestrogen-treated cells, suggesting that *trans*-tamoxifen independently regulates RNA transcription and turnover. The present study provides new rationale for the choice of molecular probes to study *trans*-tamoxifen effects on synthesis and turnover of specific nuclear and cytoplasmic RNA species.

Tamoxifen [*trans*-1-(*p*-β-dimethylaminoethoxyphenyl) 1,2-diphenylbut-1-ene] is an antiestrogen that produces clinical remissions in the majority of patients with ER-positive breast cancer (1). Although much is known about its pharmacology and metabolism, receptor binding kinetics, effects on intracellular nucleotide pools, and DNA synthesis, very little is known about tamoxifen's effects on RNA metabolism (2). Early studies measured the activities of RNA polymerases I, II, and III as well as RNA precursor incorporation into estrogen-dependent tissues shortly following tamoxifen administration (3, 4). However, it is now recognized that estrogen-like agonistic effects occur early after *in vivo* administration of tamoxifen, and its ER-antagonistic and growth-inhibiting properties become manifest only after weeks of chronic administration (5). More recently, cultured human breast cancer cells have been used to investigate the biochemical basis of tamoxifen's antitumor effects *in vitro* (6). To date, studies have confirmed that tamoxifen can antagonize estrogen-induced stimulation of known mRNA species (7, 8), but these findings do not explain how tamoxifen arrests tumor cell growth and produces clinical remissions (9). We have investigated this question by comparing the nonsteroidal antiestrogenic isomer, *trans*-tamoxifen, with its inactive *cis*-tamoxifen isomer, and with the classical steroid agonist, E_2. By measuring the effects of these agents on radioactive precursor incorporation into nuclear and cytoplasmic RNA in ER-positive human breast cancer cells, we hope to identify induction/inhibition of specific classes of ribosomal, pre-ribosomal, or low molecular weight RNAs associated with the antiestrogenic and antitumor activity of tamoxifen, independent of receptor binding differences between steroidal and nonsteroidal agents (10).

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Methods

The ER-positive human breast carcinoma T47-D cells were maintained as monolayer stock cultures in 5% CO_2 incubators at 37° using RPMI 1640 media (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT) and 0.2 IU/ml insulin (Sigma Chemical Co., St. Louis, MO). Cells were passaged in media containing 10% charcoal-stripped fetal calf serum for 1 week prior to treatment and assays. The tamoxifen isomers were supplied by Imperial Chemical Industries (Chesire, England) (*cis* = I.C.I. 47,699; *trans* = I.C.I. 46,474). E_2 (Sigma) and [5-^{3}H]Cyd (Moravek Biochemicals, Brea, CA; 26 Ci/mmol) were commerically obtained. For assessment of growth inhibition, cells (45×10^3) were plated into replicate 25-cm^2 sterile plastic flasks (Costar, Cambridge, MA) and allowed to attach for 24 hr prior to drug addition. Cells were counted at three time points during exponential growth of control cultures, and the results were expressed as mean (±standard deviation) per cent of the control cell count (11, 12). To extract radiolabeled cytoplasmic and nuclear RNA, 10^6 cells per condition were harvested by treatment with trypsin after 72 hr exposure to tamoxifen or E_2, and immediately following 2–24 hr exposure to [^{3}H]Cyd, as previously described (13).

ABBREVIATIONS: ER, estrogen receptor; E_2, 17β-estradiol.

Freshly harvested cells were washed with iced phosphate-buffered saline and gently lysed in buffered detergent containing vanadyl-ribonucleoside complex (Bethesda Research Laboratories, Rockville, MD) yielding cytoplasmic and nuclear fractions of high purity (14). After the samples were extracted with phenol/chloroform, the cytoplasmic and nuclear RNAs were isolated and purified by Proteinase K and DNase I treatments, followed by ethanol precipitation (14). These purified RNA samples from each condition were fractionated by polyacrylamide gel electrophoresis, and the gels were stained with ethidium bromide and photographed under UV light; the negatives were then scanned (600 nm) for quantitation of RNA. Incorporation (dpm) of ^3H into different size classes of RNA was determined by slicing (2 mm) the RNA bands out of the gel, incubating the slices in 0.1 N NaOH overnight, neutralizing with HCl, and assaying radioactivity by liquid scintillation counting (14). Fractional incorporation for each treatment condition was then normalized to the μg quantity of RNA loaded (as determined by scanning), and results were expressed as percentage of control, dpm/μg of RNA incorporation into each of the different size classes. Polyacrylamide (10%)/7 M urea gels were used to resolve 4–4.5S (transfer), 5S and 5.8S (ribosomal) from the other, less abundant low molecular weight nuclear and cytoplasmic RNA species (U_1, U_2, U_3, U_5, and 7S). Agarose (0.8%)/polyacrylamide (2.1%) gels were used to resolve 18S and 28S ribosomal RNA, from 20–24S and 32–45S preribosomal RNA. A Tris/borate running buffer (pH 8.0) was used for both gel conditions (14, 15). The migration pattern and resolution of the different size classes of T47-D nuclear and cytoplasmic RNA are shown in Fig. 1. Identification of the RNA classes was made according to their known migration patterns and relative nuclear and cytoplasmic abundance. Commerically obtained RNA markers were used to verify ribosomal and transfer RNA migration patterns; as well, the migration pattern of selected low molecular weight RNA species (including U_1) was previously verified by hybridization to plasmid DNA containing their coding inserts (15). Fig. 2 shows the excised gel fractions used to quantitate incorporation into the different nuclear and cytoplasmic RNA classes of interest.

Results and Discussion

The growth-inhibiting effect of a therapeutic concentration of *trans*-tamoxifen (1 μM) is shown in Fig. 3; by contrast, *cis*-tamoxifen (1 μM) and E_2 (1 nM) have no significant effect on the proliferative rate of T47-D cells under these conditions. At 1 μM concentration, the *cis*-isomer binds to ER as avidly as *trans*-tamoxifen and with a similar degree of receptor saturation as 1 nM E_2; however, *cis*-tamoxifen acts only as a weak estrogen agonist and has no antiestrogenic properties (10). The natural ER agonist, E_2, stimulates RNA and protein synthesis in these cells at nM concentrations but does not induce a significant mitogenic response (9).

After 72 hr of *trans*-tamoxifen exposure and when cell proliferation is reduced to about 50% of control, the radiolabeled T47-D RNA was extracted and fractionated. Gel resolution and pattern of migration of the nuclear and cytoplasmic RNA fractions appeared comparable for each of the treatment conditions (Fig. 1). The precursor [^3H]Cyd was chosen because it gave better incorporation into T47-D RNA than did [^3H]Urd. Under culture treatment conditions, the intervals of precursor incorporation were varied from 2 hr, 6 hr, and 24 hr, including a 6-hr pulse followed by an 18-hr chase with unlabeled precursor. Table 1 shows the treatment effects using a 24-hr [^3H]Cyd labeling interval. Although other incorporating RNA bands could be resolved on these gels, the table includes only those RNA classes showing reproducible (≤10% variation) treatment effects on ^3H labeling following replicate assay of the same RNA. These results suggest that a cytostatic dose of *trans*-tamoxifen can increase [^3H]Cyd incorporation (dpm/μg) into selected classes of cytoplasmic and nuclear RNA by as much as 6-fold when compared to control cells, or when compared to cells treated with E_2 or *cis*-tamoxifen. *cis*-Tamoxifen and E_2 treatments resulted in opposite and less significant changes in

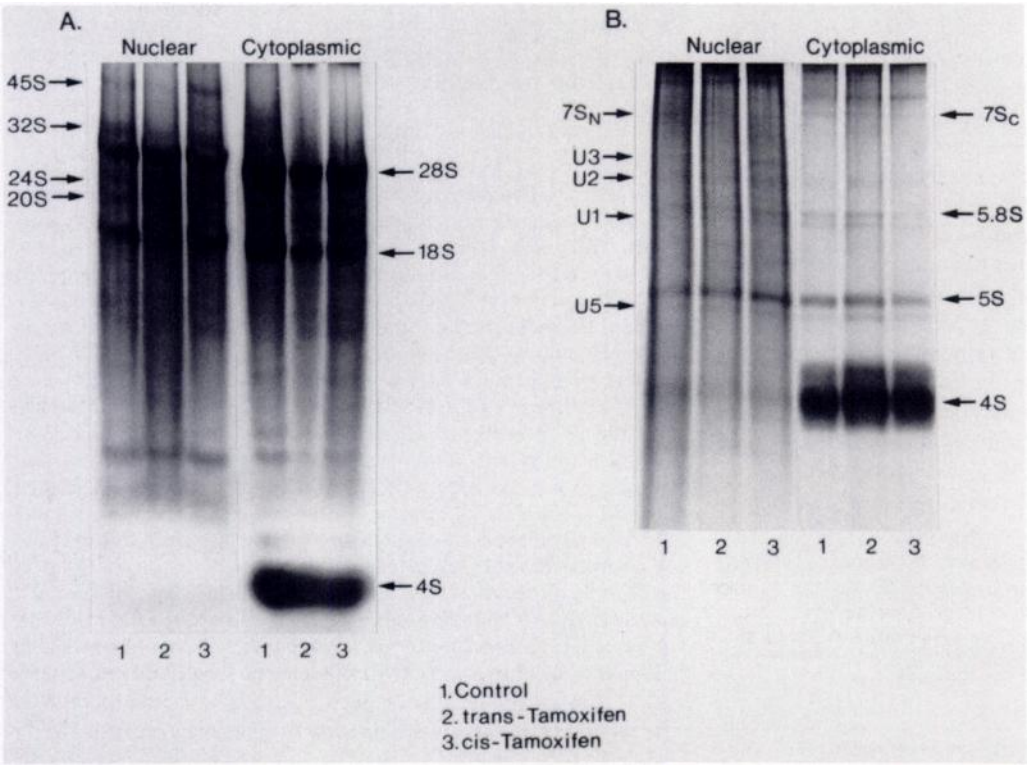

Fig. 1. Gel electrophoretic fractionation of nuclear and cytoplasmic T47-D RNA. Purified nuclear or cytoplasmic RNA (about 100 μg) from control, *cis*-, or *trans*-tamoxifen-treated (1 μM × 72 hr) cells was fractionated on both agarose (0.8%)/polyacrylamide (2.1%) (A) and polyacrylamide (10%)/7 M urea (B) gels. Gels were stained with ethidium bromide and photographed under UV illumination to observe each of the RNA bands, identified as described in the text. Negatives were scanned (600 nm) to quantitate RNA in each lane.

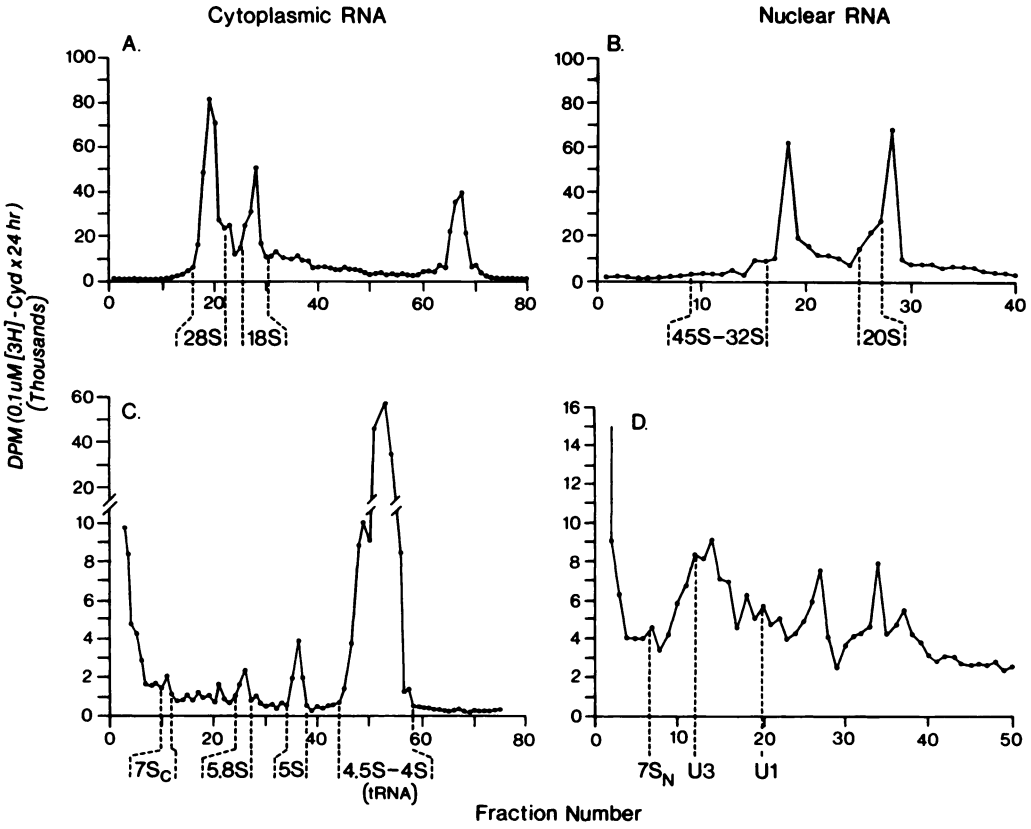

Fig. 2. Precursor incorporation into T47-D nuclear and cytoplasmic RNA classes. Gel electrophoresis, as shown in Fig. 1, was used to fractionate and identify cytoplasmic (A, C) and nuclear (B, D) RNA classes radiolabeled for 24 hr during each treatment condition (control above). RNA was isolated from gel slices (2 mm) by alkaline hydrolysis, and fractional incorporation (DPM) was determined by liquid scintillation counting. Peak areas corresponding to the appropriate ethidium bromide-stained bands were designated only for those RNA classes showing reproducible incorporation on replicate assay.

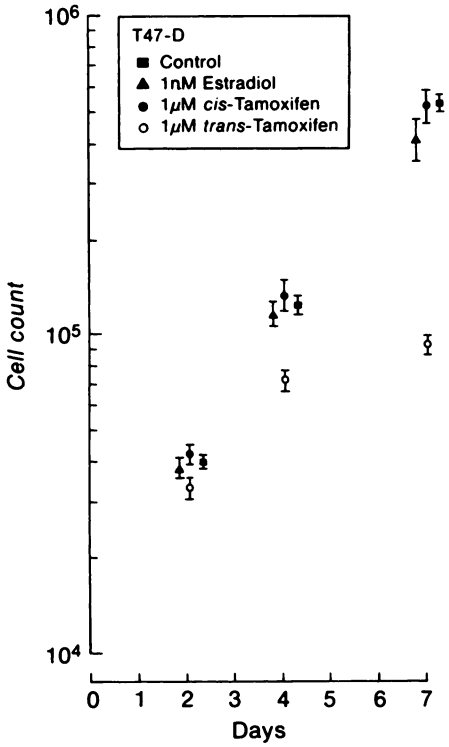

Fig. 3. Effects of treatment on culture growth of T47-D breast cancer cells. Cells (45×10^3) were plated into replicate flasks on day 0, drug or control vehicle was added after 24 hr, and cells were counted at the indicated time points during continuous culture.

TABLE 1

Antiestrogen and estrogen effects on T47-D RNA incorporation

RNA species	[^{3}H]Cyd incorporation[a]			
	Control	Percentage of Control		
		transTAM	cisTAM	E_2
	dpm × 10^{-3}	dpm/μg RNA		
Cytoplasmic				
28S	243.3	119	157	185
18S	120.6	83	91	80
$7S_c$	5.3	316	108	156
5.8S	5.6	207	90	102
5S	8.7	272	140	100
4–4.5S	210.7	317	110	112
Nuclear				
32–45S	39.4	141	71	96
20S	23.8	395	123	89
$7S_n$	6.5	339	82	113
U_3	2.6	656	120	62
U_1	2.5	360	92	88

[a] Cells passaged in media containing stripped serum were treated for 72 hr with *cis*- or *trans*-tamoxifen (1 μM), E_2 (1 nM), or control vehicle; [^{3}H]Cyd (0.1 μM × 24 hr) was added just prior to harvest and RNA was extracted and quantitated as described in Methods.

precursor incorporation, consistent with their agonistic activity.

Shorter labeling intervals (2–6 hr) produced patterns of precursor incorporation consistent with the known effects of these agents on soluble intracellular nucleotide pool sizes (16). With a 2-hr labeling interval, *trans*-tamoxifen marginally reduced incorporation into all detectable RNA classes, whereas E_2 increased incorporation into ribosomal and transfer RNA; as well, there was little detectable incorporation into the low molecular weight RNA species including U_1, U_3, $7S_c$, and $7S_n$.

With a 6-hr labeling period, all RNA classes incorporated [^{3}H] Cyd, and *trans*-tamoxifen treatment increased incorporation into the U_1 (260%), U_3 (200%), and $7S_{c+n}$ (130%) RNAs. These latter results are in keeping with the 3- to 6-fold increases detected with the 24-hr labeling interval, which is less affected by immediate changes in nucleotide pools. A 6-hr pulse of [^{3}H] Cyd chased with 18 hr of unlabeled precursor produced control levels of radionuclide detected in U_1, U_3, and 7S RNA of antiestrogen-treated cells, suggesting that *trans*-tamoxifen enhanced the turnover of these particular RNAs. The data in Table 1, as well as those from the pulse-chase experiments, also suggest that *trans*-tamoxifen partially blocked ribosomal RNA processing, resulting in decreased incorporation into 18S rRNA and increased incorporation into 20S and 32–45S pre-rRNA. A close linkage between the stimulated synthesis of pre-rRNA, U_1, U_3, and 7S RNA has recently been described in mammalian cells (17). Very little attention has been paid to the possible effects of growth-inhibiting agents on these less abundant, low molecular weight RNA classes, although the stimulatory effect observed with *trans*-tamoxifen does not appear to be a general property of growth-inhibiting drugs (15). Our preliminary results are significant in that they represent the first reported evidence that antiestrogens can potentially increase the accumulation of growth-regulating RNA species (U_1, U_3, 7S).

We previously reported that *trans*-tamoxifen reduces overall transcription and intracellular content of T47-D RNA by about 15% (13). The antiestrogen-induced increase in 24-hr precursor incorporation (dpm/μg of RNA) into various RNA classes, as illustrated in Table 1, is a new finding that could possibly result from the independent modulation of RNA catabolism and transcription by *trans*-tamoxifen. Cytostatic doses of *trans*-tamoxifen are known to paradoxically stimulate synthesis of some growth-regulating gene products (18, 19). Prior results also support the suggestion that *trans*-tamoxifen alters turnover of total T47-D RNA (13); however, its effect on turnover of specific RNA species has not been investigated. In Fig. 1 there is an apparent reduction in pre-rRNA levels in the antiestrogen-treated cells, with little change in the levels of U_1, U_3, or 7S, despite the increased labeling patterns in these RNA classes as shown in Table 1. Enhanced incorporation into pre-rRNA could be associated with a net reduction in μg of 32–45S RNA if *trans*-tamoxifen enhanced RNase activity without equally stimulating RNA polymerase I. Similarly, no net change in cellular content of U_1, U_3, or 7S RNA would be observed if antiestrogen stimulated both RNase and RNA polymerase III equally. Our observations are most consistent with the possibility that there are different antiestrogen-sensitive points of control over RNA metabolism in these breast cancer cells. Thus, it remains to be shown whether the observed increases in T47-D RNA incorporation induced by *trans*-tamoxifen actually result from selective increases in RNA synthesis, RNA turnover, or both. There is current interest in developing cDNA probes for each of the specific species of low molecular weight RNAs that may be involved in transcriptional and translational control mechanisms ($7S_k$, 5.8S, 5S), mRNA and rRNA processing (U_1, U_3), and intracellular protein transport and catabolism ($7S_L$, 4–4.5 S) (20). The availability of such probes will provide new and better methods of addressing the important questions presented by our findings. Although current methods of precursor incorporation have formed the basis for our previous understanding of RNA metabolism, these techniques are limited by high background labeling and the potential effects of treatment on intracellular precursor pools and gel migration patterns (15, 16). Nonetheless, the present study provides new rationale and direction for the choice of specific probes enabling the exact quantitation of antiestrogen-induced changes in RNA metabolism. Furthermore, the methodology illustrates the value of using equimolar doses of the nonsteroidal *cis* isomer as a control for *trans*-tamoxifen activity in assessing *in vitro* antiestrogen effects on specific classes of breast cancer RNA.

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