Effects of Tamoxifen and 4-Hydroxytamoxifen on the pNR-1 and pNR-2 Estrogen-regulated RNAs in Human Breast Cancer Cells*

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The estrogenic and antiestrogenic activities of tamoxifen and 4-hydroxytamoxifen have been measured on the expression of two estrogen-regulated RNAs (pNR-1 and pNR-2) in the MCF, human breast cancer cell line cultured in phenol red-free medium. The two antiestrogens increased the level of the pNR-1 RNA to about 80% of the estradiol-induced level, and the induction by estradiol was not significantly antagonized by either antiestrogen. In contrast, the pNR-2 mRNA was only increased to about 10% of the estradiol-induced level, and its induction by estradiol was antagonized by both tamoxifen and 4-hydroxytamoxifen. Thus, the two RNAs respond in dramatically different ways to these antiestrogens.

4-Hydroxytamoxifen and estradiol have similar affinities for the estrogen receptor; however, the induction of both RNAs by 4-hydroxytamoxifen required a 10-fold higher concentration than estradiol for maximum agonist activity, and a 500-fold molar excess was required to antagonize the induction by estradiol. Tamoxifen has a 20–100-fold lower affinity than estradiol for the estrogen receptor. A 200-fold higher concentration was required for maximum agonist activity and a 10,000-fold molar excess to antagonize the induction by estradiol.

These results emphasize the complexity of antiestrogen action in human breast cancer cells.

Human breast cancer is often estrogen-responsive; approximately one-third of breast cancer patients respond to endocrine therapy (Patterson et al., 1982). The nonsteroidal antiestrogen tamoxifen is currently widely used in the treatment of advanced breast cancer (Furr and Jordan, 1984) and increasingly in the adjuvant treatment of primary breast cancer. Although tamoxifen is widely used, its mechanism of action is poorly understood.

Triphenylethylene derivatives such as tamoxifen and its metabolite 4-hydroxytamoxifen are thought to antagonize the effects of estrogens by competing for binding to the estrogen receptor protein (Wakeling and Slater, 1981). Tamoxifen is, however, a partial estrogen agonist, but the degree of agonism varies with the tissue, species, and response being studied (Furr and Jordan, 1984). To understand the clinical effects of tamoxifen in human breast cancer, the effects and molecular basis of antiestrogen action must, therefore, be defined in human breast cancer cells.

To this end, we have isolated several estrogen-regulated RNAs from the MCF, (Soule et al., 1973) and ZR 75 (Engel et al., 1978) human breast cancer cell lines (May and Westley, 1986; Westley and May, 1987). The pNR-1 and pNR-2 RNAs are induced by estrogen and were originally isolated from MCF cells. The pNR-1 cDNA has not been completely sequenced but does not correspond to any known sequence in existing data banks. The sequence of the pNR-2 cDNA, whose RNA is identical to the pS2 RNA cloned by Masiakowski et al. (1982), suggests that the protein encoded by the RNA may be related to insulin and insulin-like growth factor 1 (Jakowlew et al., 1984). This protein may, therefore, exert hormonal activity on breast cancer or other cells.

Evaluation of the agonist activity of antiestrogens has been hampered by difficulties in obtaining cells properly withdrawn from the estrogenic compounds present in normal culture medium. It has recently been shown that phenol red, the pH indicator dye, has estrogenic activity (Berthois et al., 1986). Culture in phenol red-free media permits better withdrawal from estrogen stimulation and hence a more sensitive analysis of the agonist activity of antiestrogens.

In this study, we have examined the effects of tamoxifen and 4-hydroxytamoxifen on pNR-1 and pNR-2 RNA levels in phenol red-free medium. Both tamoxifen and 4-hydroxytamoxifen increase pNR-1 and pNR-2 RNA levels. However, while the antiestrogens induce pNR-1 almost as effectively as estradiol, pNR-2 is only induced to about 10% of the maximal level attained by estrogen.

**MATERIALS AND METHODS**

**Cell Culture—MCF:** (Soule et al., 1973), T47D (Keydar et al., 1979), and ZR 75 (Engel et al., 1978) cells were maintained in Dulbecco’s modified Eagle’s medium containing 10–15% fetal calf serum and 1 µg/ml insulin. Cells were plated into T25 or T75 flasks, grown to 80–90% confluence, and then withdrawn by culture for 5–7 days in phenol red-free modified Eagle’s medium containing 10% newborn calf serum treated with dextran-coated charcoal to remove endogenous steroids and 1 µg/ml of insulin. During the first 3 days of the withdrawal, the cells were washed twice prior to the medium change. Withdrawn cells were then cultured continuously in the above medium, whereas treated cells were cultured in the above medium supplemented with the indicated hormone. During withdrawal and treatment, culture medium was changed daily.

**RNA Preparation, Electrophoresis, and Transfer to Nitrocellulose—**

Total RNA was prepared by the method of Auffray and Rougeon (1980). RNA samples were denatured for 20 min at 65 °C in 50% dimethyl sulfoxide, 2.2 M formaldehyde, 10 mM sodium phosphate (pH 7), 0.5 mM EDTA, and 0.004% bromophenol blue. 0.5–20 µg samples were then electrophoresed through 1.2% agarose gels containing formaldehyde for 400–500 V h (Goldberg, 1980). The RNA was stained with ethidium bromide, visualized on a transilluminator, and then transferred to nitrocellulose by the method of Southern (1975). Filters were then baked in vacuo for 4 h at 80 °C.

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Labeling of RNA Probes and Hybridization—The cDNA sequences indicated by bars under their restriction maps in Fig. 1 were subcloned into the commercially available vector, pGEM 1 (Promega Biotec). The multiple cloning site contained within this vector is flanked by promoters for SP6 and T7 RNA polymerases. Radiolabeled probes were synthesized by transcription and then hybridized to the immobilized RNA at 65°C in solutions containing 50% formamide (Melton et al., 1984). The filters were washed in 0.1X SSC at 75°C. The amount of radiolabeled probe hybridized was quantified by densitometric scanning of the autoradiographs.

RESULTS

The pNR-1 and pNR-2 estrogen-regulated mRNAs were originally isolated from an MCF; cDNA library (May and Westley, 1986). 15,000 recombinants have now been screened by differential hybridization for estrogen-regulated sequences. Four recombinants containing DNA complementary to the pNR-1 RNA and 30 to the pNR-2 RNA have been isolated. The composite restriction maps of both are shown in Fig. 1. 770 nucleotides of the pNR-1 mRNA are contained within the four recombinants isolated, while almost the complete mRNA of the shorter and more abundant pNR-2 RNA was cloned.

Preparation of High Specific Activity RNA Probes—The pNR-2 mRNA is readily detected in total RNA prepared from estrogen-stimulated MCF; cells using nick-translated pNR-2 cDNA. The levels of the pNR-1 RNA are about 10-fold lower, and the levels of both RNAs are dramatically decreased in cells withdrawn from estradiol. This is particularly pronounced in cells cultured in medium lacking phenol red. To measure pNR-1 and pNR-2 RNA levels in total RNA preparations from withdrawn cells, it was necessary to increase the sensitivity from that obtained with the nick-translated probes.

cDNA corresponding to both RNAs was, therefore, subcloned into the vector pGEM 1 (Melton et al., 1984). The regions of the two RNAs that were subcloned are shown by the bars below the restriction maps in Fig. 1. The arrows indicate the direction of synthesis of the RNA probes used to detect the pNR-1 and pNR-2 mRNAs. Use of these probes increases the sensitivity of the hybridization at least 50-fold.

Agonist Effect of Tamoxifen on pNR-1 and pNR-2 RNA Levels—In a preliminary experiment, 10^{-7} M estradiol, 10^{-8} M tamoxifen, or 10^{-8} M 4-hydroxytamoxifen were added to MCF; cells withdrawn from steroids in phenol red-free medium containing 10% newborn calf serum treated with dextran-coated charcoal. Total RNA was prepared from the cells, and 2 μg were separated by agarose gel electrophoresis before transfer to nitrocellulose and hybridization with the RNA probes described above.

The results are shown in Fig. 2. The three pNR-1 RNAs of 1200, 1800, and 3500 were induced 13-fold by estradiol in the phenol red-free medium compared with an induction of around 8-fold reported previously in medium containing phenol red (May and Westley, 1986). This reflects a lower pNR-1 RNA level in the withdrawn cells. Tamoxifen and 4-hydroxytamoxifen increased pNR-1 RNA levels (11- and 10-fold) to almost the concentration obtained with estradiol.

 Estradiol increased pNR-2 RNA levels 124-fold in the phenol red-free medium compared with the 10-fold stimulation obtained previously (May and Westley, 1986). In phenol red-free medium, tamoxifen and 4-hydroxytamoxifen were partial agonists for the induction of the pNR-2 RNA. Tamoxifen induced the pNR-2 mRNA to 11% and 4-hydroxytamoxifen to 5% of the level obtained with estradiol.

Time Course of the Induction—The pNR-1 and pNR-2 RNA levels were measured in cells that had been withdrawn and then cultured for various lengths of time in the above concentrations of tamoxifen, 4-hydroxytamoxifen, and estradiol. The time courses of the inductions are shown in Fig. 3. Estradiol increased pNR-1 RNA levels within 30 min. After 24 h of treatment, the RNA was maximally increased 35-fold. This induction, although greater, is marginally slower than reported previously (May and Westley, 1986). The improved withdrawal of the cells may account for the slower response.

There was a lag in the response to both tamoxifen and 4-hydroxytamoxifen. The pNR-1 RNA levels were only twice those in withdrawn cells after 1 h of treatment but were increased 14-fold after 4 h and were maximal by 24 h. In this experiment, the antiestrogens induced pNR-1 to about 80% of the level obtained with estradiol.

The induction of pNR-2 mRNA by estradiol occurred more slowly; an increase was first detected after 1 h, was marked after 2 h, and almost maximal after 24 h. Tamoxifen and 4-hydroxytamoxifen had no effect on pNR-2 levels after 1 h but increased them after 2 h of treatment. These two compounds then increased pNR-2 mRNA levels to a maximum, 10-15% of the level obtained with estradiol at 24 h.
The induction of pNR-1 and pNR-2 RNA levels by the antiestrogens was slower than by estrogen. In all subsequent experiments cells were treated for at least 48 h to ensure maximal RNA induction.

Concentration Dependence of the Induction of pNR-1 and pNR-2 by Tamoxifen and 4-Hydroxytamoxifen—The agonist activity of tamoxifen and 4-hydroxytamoxifen in other systems is dose-dependent. The induction of pNR-1 and pNR-2 RNAs was, therefore, measured over a range of antiestrogen concentrations. MCF-7 cells were withdrawn and then cultured in different concentrations of estradiol or one of the antiestrogens for 2 days. RNA was prepared and then hybridized with the single-stranded probes as described under "Materials and Methods." The results are shown in Fig. 4.

The induction of pNR-1 RNA levels by estradiol was half-maximal at $10^{-11}$ M. pNR-1 RNA levels were induced half-maximally by $10^{-10}$ M 4-hydroxytamoxifen or $10^{-8}$ M tamoxifen. The pNR-2 mRNA was slightly less sensitive to estrogen; the induction was half-maximal at $2 \times 10^{-11}$ M and maximal by $10^{-9}$ M estradiol. Concentrations of 4-hydroxytamoxifen between $10^{-10}$ and $10^{-8}$ M slightly increased pNR-2 mRNA levels. The maximum level at $10^{-9}$ M induced by 4-hydroxytamoxifen was 10% of the estrogen-induced level. Tamoxifen also increased pNR-2 mRNA levels at concentrations higher than $10^{-7}$ M.

Effect of Tamoxifen and 4-Hydroxytamoxifen on the Induction of pNR-1 and pNR-2 RNAs by Estradiol—The ability of tamoxifen and 4-hydroxytamoxifen to antagonize the induction of the two RNAs by $2 \times 10^{-10}$ M estradiol was tested. MCF-7 cells were withdrawn and then different concentrations of the antiestrogens and estradiol were added simultaneously to the culture medium. After a further 2 days of culture, total RNA was extracted from the cells and analyzed as described under "Materials and Methods."

As shown in Fig. 5, the pNR-1 RNA level is not altered by any concentration of tamoxifen between $10^{-9}$ and $10^{-6}$ M. 4-Hydroxytamoxifen decreased pNR-1 levels somewhat but did not reduce them below 70% of the maximal levels even at the highest concentrations tested. Tamoxifen and 4-hydroxytamoxifen, therefore, had little effect on the estrogen-stimulated levels of pNR-1 RNA, although at higher concentrations they reduce the pNR-1 RNA induction to the slightly lower levels obtained by the antiestrogens alone.

In contrast, both tamoxifen and 4-hydroxytamoxifen antagonized the induction of pNR-2 RNA by estradiol. The induction was partially antagonized by $10^{-6}$ M tamoxifen (5000-fold molar excess) and was almost completely inhibited by $10^{-7}$ M tamoxifen. 4-Hydroxytamoxifen inhibited the induction of pNR-2 mRNA at $10^{-7}$ M (500-fold molar excess).
In the presence of $10^{-8}$ M 4-hydroxytamoxifen, the induction of pNR-2 RNA was at the level present in withdrawn cells.  

**Effects of Tamoxifen and 4-Hydroxytamoxifen in Other Cell Lines**—To establish whether the agonist activity of tamoxifen and 4-hydroxytamoxifen is unique to MCF7 cells or a more general feature of breast cancer cell lines, the effects of tamoxifen and 4-hydroxytamoxifen on pNR-1 and pNR-2 RNA levels were also tested in the T47D (Keydar et al., 1979) and ZR 75 (Engel et al., 1978) cell lines. All three cell lines are derived from metastatic human breast cancer cells, contain estrogen receptor protein (Redell et al., 1985), and are estrogen-responsive for growth (Lippman and Bolan, 1975; Reddell and Sutherland, 1984; Darbre et al., 1984). The cells were first withdrawn and then cultured with 4 days in the withdrawal medium alone or in medium containing $10^{-8}$ M estradiol, $10^{-6}$ M tamoxifen, or $10^{-6}$ M 4-hydroxytamoxifen. Total RNA was prepared, and the pNR-1 and pNR-2 RNA levels were measured as described under "Materials and Methods." The results are shown in Fig. 6 and in Table I.  

*pNR-1* RNA is expressed and regulated by estradiol in all three cell lines. There is, however, a large variation in its level in the different estrogen-treated cells. It is most abundant in the MCF7 cell line and is present at only 0.5% of this level in the ZR 75 cells. The induction by estradiol ranged from 14-fold in the MCF7 cell line to 6-fold in the T47D cell line. Tamoxifen and 4-hydroxytamoxifen induce pNR-1 RNA in all cell lines. Their agonist activity varies from 33% of the maximum for 4-hydroxytamoxifen in the ZR 75 cells to 160% for 4-hydroxytamoxifen in the T47D cells.  

*pNR-2* RNA is also expressed and induced by estrogen in all three human breast cancer cell lines. The relative levels of its mRNA in the different cell lines are as variable as those of pNR-1. It is most abundant in the MCF7 cells and least abundant in the T47D cell line where it is expressed at approximately 0.5% of the level in MCF7 cells. The extremely low level of pNR-2 mRNA expression in T47D cells explains why it has previously been reported to be absent from these cells (Brown et al., 1973).
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et al., 1984; May and Westley, 1986). The induction of the pNR-2 mRNA by estradiol was greatest in the MCF cells (50-fold) and least in the ZR 75 cells (30-fold). The two antiestrogens are weak partial agonists for the induction of the pNR-2 RNA in the three cell lines. The largest induction was to 19% of the estrogen-induced level by tamoxifen in the T47D cell line.

**DISCUSSION**

Although tamoxifen is widely used in the treatment of human breast cancer, the molecular basis of antiestrogen action in these cells is not yet understood. Breast cancer cell lines have been established that retain steroid receptors and hormone responsiveness (e.g. Soule et al., 1973), and these cell lines, therefore, provide useful experimental systems for analyzing the mechanism of action of antiestrogens. Recently, we have cloned several estrogen-responsive RNA sequences from breast cancer cells, and the corresponding cDNAs can, therefore, be used to study the effects of antiestrogens on specific estrogen-regulated mRNAs (May and Westley, 1986).

We have examined the effect of tamoxifen and a hydroxylated metabolite on two such RNAs. In MCF cells, these antiestrogens act as weak partial estrogens for the induction of pNR-2, whereas for pNR-1, both antiestrogens are almost fall estrogen agonists. Their induction of pNR-2 is maximally about 10% of that observed using estradiol and is dose-dependent with the maximum induction being observed at $10^{-8} M$ for 4-hydroxytamoxifen and at $10^{-7} M$ for tamoxifen. We have previously reported (Westley et al., 1984) that tamoxifen does not induce the pS2 RNA cloned by Masiakowski et al. (1982) that is identical to the pNR-2 RNA cloned by ourselves. It is probable that the discrepancy between the two studies results from the use of phenol red-free medium (Berthois et al., 1986) in this study. Phenol red is a weak estrogen, and in its absence, the levels of the estrogen-regulated RNAs in withdrawn cells are much lower than in conventional medium containing phenol red. Thus, we are now able to obtain up to a 100-fold induction of the pNR-2 RNA by estrogen whereas we previously typically obtained a 10-fold induction (May and Westley, 1986). Berthois et al. (1986) have observed the same phenomenon for the proliferation of MCF cells; in medium containing phenol red, tamoxifen acted as a pure estrogen antagonist whereas in phenol red-free medium, in which the rate of proliferation of withdrawn cells was much lower, tamoxifen and 4-hydroxytamoxifen both slightly increased cell proliferation.

The induction of pNR-2 by the two antiestrogens was dose-dependent, 4-hydroxytamoxifen giving a bell-shaped curve. Tamoxifen was more agonist at $10^{-8} M$ and 4-hydroxytamoxifen at $10^{-7} M$. Biphasic dose-response curves at antiestrogen concentrations have been observed previously for the induction of the progesterone receptor by tamoxifen in MCF cells (Horwitz et al., 1978) and the proliferation of T47D cells (Reddel and Sutherland, 1984). In both cases, tamoxifen had a maximum effect at $10^{-7} M$.

These dose-response curves are difficult to interpret. It is unlikely that the binding of a ligand to a single site on a receptor could generate a biphasic curve. It could result, however, from interactions of the ligand with two different binding sites within the cell, binding to the higher affinity site causing an increase in pNR-2 levels and binding to the lower affinity site resulting in the lowering of pNR-2 levels seen at higher concentrations.

High affinity binding sites ($K_d 1 nM$) distinct from the estrogen receptor have been identified for triphenylethylen derivatives such as tamoxifen (Sutherland et al., 1980; Millar and Katzenellenbogen, 1983), and it has been postulated that these sites might be involved in mediating the effects of antiestrogens. The interaction of tamoxifen and 4-hydroxytamoxifen with antiestrogen binding sites and the estrogen receptor may provide a better explanation of the action of antiestrogens than an interaction with the estrogen receptor alone. However, the relevance of these binding sites to the effects of antiestrogens on gene expression and cell proliferation has been questioned (Sheen et al., 1985).

Estradiol and 4-hydroxytamoxifen have a similar affinity for the estrogen receptor ($K_d 0.2 nM$) whereas the affinity of tamoxifen is at least 20-fold lower. There is, therefore, a discrepancy between the relative affinities of the compounds for the estrogen receptor and the concentrations required to increase pNR-1 and pNR-2 RNA levels: 10-fold more 4-hydroxytamoxifen than estradiol is required.

Both tamoxifen and 4-hydroxytamoxifen antagonize the induction of pNR-2 by estradiol, a 200- and 10,000-fold molar excess of 4-hydroxytamoxifen and tamoxifen, respectively, being required to reduce the induction by $2 \times 10^{-10} M$ estradiol by 50%. The concentrations of tamoxifen and 4-hydroxytamoxifen that are required to antagonize the effects of estradiol are, therefore, much greater than would be expected from both their relative affinities for the estrogen receptor and their potencies as estrogen agonists.

Our finding that tamoxifen and 4-hydroxytamoxifen have similar effects on the pNR-1 and pNR-2 RNAs but at different concentrations is compatible with the model of Jordan and Liberman (1984). This model suggests that the alkyl aminothioxy side chain common to both compounds produces the specific conformational perturbation of the receptor that determines the biological activity of the antiestrogen-receptor complex for a particular response. The affinity of the compound for the receptor determines the concentration at which it is effective. The hydroxylation of tamoxifen to give 4-hydroxytamoxifen increases its affinity for the estrogen receptor and renders it active at a lower concentration.

Our results also suggest that the mechanism of action of antiestrogens in breast cancer cells is more complex than suggested by this model. Previous studies have generally focused on a single response, e.g. activation of the estrogen receptor (Rochefort and Borgna, 1981), the synthesis of specific proteins (Westley and Rochefort, 1980; Liberman et al., 1983), or cell proliferation (Reddel et al., 1985). There have been very few reports in which the effects of tamoxifen have been measured on specific RNA levels, and this is the first study to our knowledge in which the levels of two specific RNAs have been measured. The simplest models for antiestrogen action focus on the estrogen receptor and generally suggest that the antiestrogen competes with estradiol for binding to its receptor, thereby resulting in an inactive or partially active antiestrogen-estrogen receptor complex (Liberman et al., 1983).

Our finding that both tamoxifen and 4-hydroxytamoxifen have significantly different estrogen agonist activity for the induction of pNR-1 and pNR-2 suggests that this type of model is inadequate and that there must be an additional level of discrimination. The estrogen receptor is thought to act by binding to regulatory sequences within responsive genes, thereby modulating their transcription (Klein-Hitpass et al., 1986). One possibility is that for some genes such as the pNR-2, a tamoxifen estrogen-receptor complex is not able to bind to these control regions in such a way as to increase expression whereas for other genes such as pNR-1, the controlling sequence cannot discriminate between the two types of complex. This model which, therefore, emphasizes the role
of the control regions of particular genes would allow for a spectrum of estrogen agonism depending on the way that the regulatory DNA sequences interpreted the structure of the antiestrogen-estrogen receptor complex. The cloning of the genes coding for pNR-1 and pNR-2 RNAs and the identification and characterization of the regulatory sequences should provide direct experimental evidence for this type of model.

Tamoxifen has proved to be clinically important for the treatment of advanced breast cancer, and it is probable that this effectiveness is due to its antiestrogenic properties (Furr and Jordan, 1984). It has been suggested that tamoxifen may be effective in adjunct therapy for primary breast cancer and even given prophylactically to women with a high risk of developing breast cancer (Cuzick et al., 1986). Our results show that the response to tamoxifen is not easily predicted and that care should be taken not to extrapolate the results obtained using tamoxifen for the treatment of advanced breast cancer to other clinical situations. They also highlight the need for a new generation of antiestrogens that completely lack estrogenic activity.

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