Pharmacological Relevance of Endoxifen in a Laboratory Simulation of Breast Cancer in Postmenopausal Patients

Philipp Y. Maximov, Russell E. McDaniel, Daphne J. Fernandes, Puspanjali Bhatta, Valeriy R. Korostyshevskiy, Ramona F. Curpan, V. Craig Jordan

Manuscript received December 13, 2013; revised July 24, 2014; accepted August 1, 2014.

Correspondence to: V. Craig Jordan, OBE, PhD, DSc, FMedSci, Oncology and Pharmacology, Georgetown University Medical Center, 3970 Reservoir Rd NW, Research Building, Suite E501, Washington, DC 20057 (e-mail: vcj2@georgetown.edu).

Background

Tamoxifen is metabolically activated via a CYP2D6 enzyme system to the more potent hydroxylated derivatives 4-hydroxytamoxifen and endoxifen. This study addresses the pharmacological importance of endoxifen by simulating clinical scenarios in vitro.

Methods

Clinical levels of tamoxifen metabolites in postmenopausal breast cancer patients previously genotyped for CYP2D6 were used in vitro along with clinical estrogen levels (estrone and estradiol) in postmenopausal patients determined in previous studies. The biological effects on cell growth were evaluated in a panel of estrogen receptor–positive breast cancer cell lines via cell proliferation assays and real-time polymerase chain reaction (PCR). Data were analyzed with one- and two-way analysis of variance and Student's t test. All statistical tests were two-sided.

Results

Postmenopausal levels of estrogen-induced proliferation of all test breast cancer cell lines (mean fold induction ± SD vs vehicle control: MCF-7 = 11 ± 1.74, P < .001; T47D = 7.52 ± 0.72, P < .001; BT474 = 1.75 ± 0.23, P < .001; ZR-75-1 = 5.5 ± 1.95, P = .001. Tamoxifen and primary metabolites completely inhibited cell growth regardless of the CYP2D6 genotype in all cell lines (mean fold induction ± SD vs vehicle control: MCF-7 = 1.57 ± 0.38, P = .54; T47D = 1.17 ± 0.23, P = .79; BT474 = 0.96 ± 0.2, P = .98; ZR-75-1 = 0.86 ± 0.67, P = .99). Interestingly, tamoxifen and its primary metabolites were not able to fully inhibit the estrogen-stimulated expression of estrogen-responsive genes in MCF-7 cells (P < .05 for all genes), but the addition of endoxifen was able to produce additional antiestrogenic effect on these genes.

Conclusions

The results indicate that tamoxifen and other metabolites, excluding endoxifen, completely inhibit estrogen-stimulated growth in all cell lines, but additional antiestrogenic action from endoxifen is necessary for complete blockade of estrogen-stimulated genes. Endoxifen is of supportive importance for the therapeutic effect of tamoxifen in a postmenopausal setting.


Tamoxifen, a nonsteroidal antiestrogen, is a pioneering therapy for the treatment of breast cancer targeted to the tumor estrogen receptor (ER) (1). The laboratory strategy (2) of targeting tumor ER with long-term antihormone therapy accurately translated to clinical practice with enhanced control of disease recurrence following long-term adjuvant therapy in both pre- and postmenopausal patients. This targeted adjuvant treatment strategy ultimately resulted in a 30% decrease in mortality (3–6). Indeed, long-term (five years) adjuvant tamoxifen therapy contributes substantially to national statistics with the reduction in death rate from breast cancer during the past decade (7).

Early findings demonstrated that tamoxifen is metabolically activated to 4-hydroxytamoxifen (Figure 1) that has a hundred-fold increase in affinity for the ER (8,9). However, high affinity of a ligand for the ER is an advantage but not a requirement for antiestrogenic activity (10,11). Indeed, studies comparing the antitumor activity of tamoxifen and 4-hydroxytamoxifen (4OHT) in vivo demonstrated that tamoxifen was the superior agent for clinical development (10). 4-Hydroxytamoxifen is cleared from the body faster than tamoxifen while the parent drug tamoxifen accumulates (12). Nevertheless, the subsequent identification of 4-hydroxy-N-desmethyltamoxifen (13–15) (later called endoxifen) (Figure 1) and the demonstration of a reduction of endoxifen production in women taking Selective Serotonin Reuptake Inhibitors (SSRI) (for reduction of hot flashes), which block the enzymatic activity of CYP2D6 (16), suggested that this important drug interaction reduces tamoxifen’s efficacy. Subsequently, a hypothesis was developed and connected with genotypic aberrations in CYP2D6 alleles (17). The hypothesis depends on the concept that different
polymorphisms of CYP2D6 can result in altered enzymatic activity of the cytochrome P450 2D6, which results in different rates of tamoxifen metabolism and thus different levels of tamoxifen metabolites N-desmethyltamoxifen (NDMTAM) and endoxifen. Some clinical studies have shown association between the CYP2D6 genotypes and the clinical outcomes (18–20), and some have not found any association (21–23). Numerous papers using retrospective clinical data presented convincing cases for or against the hypothesis. However, the low incidence of poor metabolizers (PM) (24), and the known poor compliance rate (25) of long-term antihormone therapies make the hypothesis difficult to validate in retrospective patient samples.

In this article we address the role of the CYP2D6 genotype during tamoxifen treatment of breast cancer in a laboratory simulation. We have characterized a panel of four representative ER-positive breast cancer cell lines, MCF-7, T47D, BT474, and ZR-75-1, and carefully calibrated their concentration responsiveness to both estradiol (E$_2$) and estrone (E$_1$). We selected concentrations of total estrogen (E$_1$/E$_2$) corresponding to the previously reported circulating levels in postmenopausal breast cancer patients (26,27). We address two hypotheses: 1) that tamoxifen is a prodrug that needs to be metabolically activated to NDMTAM and 4OHT, and 2) that circulating concentrations of tamoxifen and its two metabolites NDMTAM and 4OHT are sufficient to block estrogen-stimulated breast cancer cell growth and gene expression in a postmenopausal patient simulation. Our chosen concentrations of tamoxifen and metabolites have been determined recently for postmenopausal patients genotyped for CYP2D6 as extensive metabolizers (EM), intermediate metabolizers (IM), and PM (28). The experiment was repeated with all cell lines to address the second hypothesis with and without relevant concentrations of endoxifen for each genotype. Overall, metabolic activation to endoxifen appears to play a supportive role in blocking estrogen-induced cell replication in breast cancer cell lines in a simulated model of postmenopausal breast cancer. Conversion to endoxifen is perhaps consolidating the long-term benefits of antiestrogen therapy but is not essential for the immediate antiestrogenic antitumor actions of tamoxifen in the postmenopausal setting.

**Methods**

**Cells and Culture Conditions**
The ER-positive human breast cancer cell lines MCF-7, T47D, BT474, and ZR-75-1 were used in this study. All cells were obtained from American Type Culture Collection (ATCC) and were maintained in phenol-red RPMI 1640 medium. All cultures were grown in 5% CO$_2$ at 37°C. All cell lines were verified using DNA fingerprinting analysis performed at the tissue culture shared resource at the Lombardi Comprehensive Cancer Center (29).

**Cell Proliferation Assays**
All cells were cultured in estrogen-free medium (phenol-red-free Roswell Park Memorial Institute 1640 media supplemented with 10% charcoal-stripped fetal bovine serum) for three days before beginning the proliferation assay. The proliferation assays were performed after a seven-day treatment as previously described (BibXref) using a fluorescent DNA quantification kit and calf thymus DNA for the standard curve. To study the pharmacological importance of endoxifen we simulated the postmenopausal estrogen setting for patients in each of our cell lines and used the actual clinical concentrations of tamoxifen and its metabolites found in patients previously genotyped for CYP2D6 and...
treated with tamoxifen (Supplementary Table 1, available online).

To simulate the clinical setting in vitro we selected a panel of ER-positive human breast cancer cell lines: MCF-7, T47D, BT474, and ZR-75-1; all were previously shown to be ER-positive (31) by western blotting and estrogen-responsive for growth. Experiments, each consisting of three replicates, were performed at least three times.

**Real-Time Polymerase Chain Reaction**

Real-time polymerase chain reaction (PCR) was performed as previously described (32). Primers sequences that were used for human pS2 cDNA amplification are: 5'-CAT CGA CGTCCCTCCAGGAAGA-3' sense, and 5'-CTCTGGGACT AATCACCCTGCTG-3' anti-sense; human GREB1 gene: 5'-CAAAGATAAACCTGTGGCCCTGC-3'sense, 5'-GA CAT GCCTGCCTCTCATACT-3' anti-sense; human proges-
teron receptor (PgR): 5'-CGTGCTATCTGCGCTCTCA-3' sense, 5'-CCGCGTGCTAATTTCCTG-3' anti-sense; the reference gene 36B4: 5'-GTGTTCGACAATGGCAGCAT-3' sense, 5'-CCGCCGTCGTAACTTTCGT-3' anti-sense; the reference gene 36B4: 5'-GTGTTCGACAATGGCAGCAT-3' sense, 5'-CACACCTCCAGGAAGCGA-3' anti-sense. All primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA). Experiments, each consisting of three replicates, were performed at least three times.

**Immunoblotting**

Immunoblotting was performed as previously described (30). The membranes were probed with rabbit polyclonal anti-ERα antibody at 1:500 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) and with mouse monoclonal anti-β-actin antibody at 1:40,000 dilution (Sigma-Aldrich, St. Louis, MO) antibodies, as recommended by the supplier. All protein levels were analyzed by densitometry using ImageJ software (NIH). Experiments, each consisting of three replicates, were performed at least three times.

**Molecular Modelling**

The experimental X-ray structures of ERα LBD to be used for docking were selected from Protein Databank (33) based on the three-dimensional shape similarity between the compounds to be docked and cocrystallized ligands extracted from the receptor-ligand complex similarity. The three-dimensional shape was computed using the Rapid Overlay of Chemical Structures utility of OpenEye. The ligands of interest were used as the query dataset while the screening library was compiled from the ligands extracted from all of the available crystal structures of human ERα deposited in the Protein Data Bank (PDB). ShapeTanimoto parameter was used for scoring with a cutoff value of 0.8, and four ligands met this criterion. The 3D coordinates of the corresponding ERα complexes were extracted from PDB entries 3ERT (34). Subsequently, the structures were prepared for docking using the Protein Preparation Workflow (Schrödinger, LLC, New York, NY, 2011) accessible from within the Maestro program (Maestro, version 9.2; Schrödinger, LLC, New York, NY, 2011). Briefly, the hydrogens were properly added to the complexes, water molecules beyond 5Å from a heteroatom were deleted, bond corrections were applied to the cocrystallized ligands, the orientation of hydroxyl groups, Asn, Glu and the protonation state of His were optimized to maximize hydrogen bond formation. All Asp, Glu, Arg, and Lys residues were left in their charged state. In the final stage, a restrained minimization on the ligand-protein complexes was carried out with the OPLS_2001 force field, and the default value for root-mean-square deviation of 0.30Å for non-hydrogen atoms was used. Docking simulations were performed with Glide software (Glide, version 5.7; Schrödinger, LLC, New York, NY, 2011), a grid-based docking method that can be run rigid or fully flexible for the ligand (35,36). To some extent, a degree of flexibility was allowed to the X-ray structures of ERα in agonist conformation by scaling down the van der Waals radii of nonpolar atoms with a scale factor of 0.8 and allowing the free rotation of hydroxyl groups.

**Statistical Analysis**

To test the interactions between treatment and genotype in the proliferation assays, we used analysis of variance (ANOVA) with a balanced two-factor design. When a statistically significant interaction was present, we used one-way ANOVA to investigate simple effects of the independent factors. A P value less than .05 was considered significant. All computations were carried out using R, Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria). For testing the significance of treatment in RT-PCR experiments, the Student’s t test was used. All statistical tests were two-sided.

**Results**

**Effects of Estrogens, Tamoxifen and Metabolites, and Endoxifen on Cell Proliferation**

Concentration response curves for all cell lines with increasing concentrations of either E₁ or E₂ are shown in Figure 2. The estrogen levels in postmenopausal patients treated with tamoxifen alone that were used are based on the calculated levels of E₁ and E₂ in patient plasma reported in the literature (26,27). The recalculated concentrations used for all assays were 7.8x10⁻¹⁰M for E₁ (which corresponds to the average 21 pg/mL in patient plasma) and 4.7x10⁻¹¹M for E₂ (which corresponds to the average 13 pg/mL in patient plasma); these concentrations combined were used in subsequent treatments of the cells to simulate the postmenopausal setting. Estrogen levels were also consistent with plasma levels reported by other groups (37,38) for postmenopausal patients. To simulate the clinical setting of postmenopausal patients treated with tamoxifen, we used concentrations of tamoxifen and all its metabolites previously published for a clinical study of different CYP2D6 genotypes (28) (Supplementary Table 1, available online). The metabolic activation of tamoxifen to endoxifen by different CYP2D6 genotypes occurs at different rates, resulting in different plasma levels of endoxifen, NDMTAM, and tamoxifen itself. 4OHT remains consistently low. Using the published concentrations of tamoxifen and its metabolites, along with the calculated concentrations of estrogens, we treated all cell lines and assessed the biological effect on growth using a seven-day proliferation assay.

All cell lines (Figure 3) were responsive to treatment with the estrogen combination (E₁/E₂) alone (mean fold induction ± SD vs vehicle control: MCF-7 = 11 ± 1.74, P < .001 [Figure 3A]; T47D = 7.52 ± 0.72, P < .001 [Figure 3B]; BT474 = 1.75 ± 0.23,
The addition of tamoxifen plus primary metabolites NDMTAM and 4OHT (TPM) to E1/E2 (Figure 3) completely inhibited estrogen-stimulated cell growth (mean fold induction ± SD vs vehicle control: MCF-7 = 1.57 ± 0.38, P = .54 [Figure 3A]; T47D = 1.17 ± 0.23, P = .79 [Figure 3B]; BT474 = 0.96 ± 0.2, P = .98 [Figure 3C]; ZR-75-1 = 0.86 ± 0.67, P = .99 [Figure 3D]; all P by one-way ANOVA).

Addition of endoxifen to the TPM and E1/E2 treatment (Figure 3, A-D, col. 6) did not produce a further antiestrogenic effect in any of the genotype groups (P = .82 for MCF-7, P = .95 for T47D, P = .95 for BT474, and P = .99 for ZR-75-1; one-way ANOVA compared with TPM + E1/E2). Assessment of endoxifen’s pharmacological impact when added to the TPM + E1/E2 treatment in all tested cell lines using a two-way ANOVA approach did not reveal any statistically significant antiestrogenic effect enhancement in any of the genotype categories, with all P values higher than .05.

To assess the importance of the active metabolite 4OHT, we performed proliferation assays in our panel of cells after treatment with postmenopausal concentrations of estrogens and estrogens in combination with tamoxifen and NDMTAM, and estrogens in combination with tamoxifen and its primary metabolites. The results demonstrate that treatment with tamoxifen plus the primary metabolite NDMTAM at concentrations corresponding to patients with EM genotype (Supplementary Table 1, available online) inhibited the estrogen stimulated growth of MCF-7 cells by 39.62 ± 2.26% (P = .02, Student’s t-test) (Figure 4A, col. 3); however, addition of 4OHT in the EM genotype concentration (Supplementary Table 1, available online) completely inhibited the proliferation action of estrogens (94.72 ± 0.06% average inhibition vs E1/E2 treatment (P = .15 vs vehicle control, Student’s t-test) (Figure 4A).

Additionally, we assessed the biological effect of tamoxifen metabolites in a postmenopausal setting in hypothetical osteoporotic women. To simulate an osteoporotic scenario, we used estrogen concentrations 10-fold lower than the average concentrations used in our study, which coincides with estrogen plasma levels in postmenopausal patients from the clinical study of osteoporotic women determined in the results for the analysis of the Multiple Outcomes of Raloxifene Evaluation study (39). The results demonstrated that treatment of the MCF-7 breast cancer cell line with a combination of tamoxifen and primary metabolites without endoxifen completely inhibit estrogen action (Supplementary Figure 1, available online).
Effect of Endoxifen on Estrogen-Stimulated Genes

To assess the estrogenic and antiestrogenic effect on the transcriptional activity of ER and estrogen-responsive gene expression in MCF-7 cells, we focused on the contribution to the overall effect of estrogen and the antiestrogenic effect of either or both of the active metabolites 4OHT and endoxifen. We performed RT-PCR as described in the Methods section and used primers for GREB1, pS2, and PgR estrogen-responsive genes. Results for all genes were similar. We demonstrated that GREB1 (Figure 4B), a regulator of hormone response in breast cancer (40), was activated 333.42 ± 58.18-fold by estrogens compared with vehicle control (P = .005, Student’s t test). Addition of tamoxifen and NDMTAM reduced the estrogenic effect by 73 ± 5.3% of E1/E2 treatment, P = .015 compared with E1/E2 treatment alone, but was statistically significantly different from vehicle control (P = .007). Addition of 4OHT in EM concentration reduced the fold change in GREB1 mRNA levels even more when compared with the tamoxifen and NDMTAM combination treatment (down to 90 ± 1.08% of E1/E2 treatment, P = .022). Addition of endoxifen to tamoxifen and its primary metabolite mix (TPM) completely inhibited the estrogenic effect, bringing the fold change down to control levels (P = .34).

Additionally, we studied the effects of these treatments on other estrogen-responsive genes, such as pS2 (Figure 4C) and PgR (Figure 4D). Estrogens induced both genes; however, in both cases, addition of tamoxifen and NDMTAM to the E1/E2 mix elevated the average mRNA expression of these genes; however, this was not statistically significant (P = .26 for pS2, and P = .24 for PgR). Addition of 4OHT to tamoxifen and NDMTAM in both genes reduced mRNA expression (78.9 ± 1.34%, P = .006 for pS2; and 91 ± 2.4%, P = .04 for PgR compared with estrogen treatment). Addition of endoxifen (EN) to tamoxifen and primary metabolites (TPM) further inhibited the estrogen-stimulated expression of mRNA.
the mRNAs but not statistically significantly vs TPM treatment ($P = .23$ for pS2, and $P = .48$ for PgR).

**Effect of Tamoxifen and Metabolites on ER$\alpha$ Protein Stability**

We assessed the effect of tamoxifen and metabolites on ER$\alpha$ protein stability after 24 hours of treatment in MCF-7 cells. Our results indicated that MCF-7 cells in a postmenopausal estrogen (E$_1$/E$_2$) environment reduced the levels of ER$\alpha$ protein by approximately 15% as measured by densitometry compared with vehicle control (Figure 5A, lane 2). Tamoxifen plus primary metabolites (TPM) at EM concentrations stabilized the levels of ER$\alpha$ by approximately 27% when compared with vehicle control as measured by densitometry. In combination with E$_1$/E$_2$, TPM reversed the effect of estrogens on ER$\alpha$, consistent with the antiestrogenic effect of tamoxifen (Figure 5A, lane 4) and stabilized the levels of the ER$\alpha$ protein by about 37% when compared with vehicle control. We compared the effects of endoxifen from different sources (Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany; Indiana School of Medicine, Indianapolis, IN; and the Mayo Clinic, Rochester, MN) in concentrations reported for patients with an EM genotype (Figure 5A). Results showed clear differences in the stabilization of the ER$\alpha$ protein, but we did not find any reduction in protein levels (Figure 5A). 4-Hydroxytamoxifen clearly stabilized the ER$\alpha$ protein levels at EM genotype concentrations by approximately 36% when compared with vehicle control. TPM and endoxifen prevented the estrogen-induced degradation of the ER$\alpha$ protein.

ICI 182780 (fulvestrant) was used as a positive control for ER degradation at a 1 µM concentration. We assessed the effects of 4OHT and endoxifen on ER$\alpha$ protein stability in MCF-7 cells at high concentrations of 1 µM with 1nM E$_2$ after 24 hours of treatment (Figure 5B). Treatment with 1nM E$_2$ treatment resulted in downregulation of the ER$\alpha$ protein by 50% compared with vehicle control as measured by densitometry. Treatments with 1 µM 4OHT alone and 1 µM endoxifen alone...
each stabilized the ERα protein by 100% and 90%, respectively, when compared with vehicle control as measured by densitometry. A combination of 1 nM E2, with either 1 µM 4OHT or endoxifen, reversed the estrogen-induced degradation of ERα (ERα protein stabilization by 92% with 4OHT and 54% with endoxifen when compared with vehicle control as measured by densitometry), showing the consistent antiestrogenic regulation of ERα protein stability with endoxifen.

Molecular Modelling of 4OHT and Endoxifen With the Ligand-Binding Domain of the ER

The superimposition of the top-ranked, induced-fit docking pose of endoxifen onto the ERα-4OHT complex shows a predicted binding alignment of endoxifen similar to that of 4OHT. The same H-bond network is recapitulated, which engages contacts between the phenolic hydroxyl group of endoxifen and E353, R394, and a crystallized water molecule. The core structures of both ligands overlap almost perfectly, the major difference being in the aminoalkyl side chain. The positioning of the flexible methylaminoethyl group of endoxifen and the side chain of D351 is shifted when compared with the 4OHT complex. Thus, the carboxylate group of D351 is moved towards the aminoethyl group, lying 2.6 Å away, compared with 3.8 Å in the case of the 4OHT complex and being involved in a salt bridge formation. This indicates that the conformation of the ER in complex with endoxifen is very similar to the known conformation of the ER-4OHT complex.

Discussion

To our knowledge, this study is the first to employ the actual circulating concentrations of tamoxifen and its metabolites measured in tamoxifen-treated postmenopausal patients and used to block estrogen action from clinically derived plasma levels using a panel of human breast cancer cell lines. We found that tamoxifen and its primary metabolites in concentrations found in three CYP2D6 genotypes (EM, IM, and PM) are sufficient to inhibit estrogen-induced replication in the postmenopausal setting. We also established the importance of 4OHT as the active primary metabolite in the antiestrogenic action of tamoxifen in the postmenopausal setting. The estrogenic steroids would be anticipated to accumulate in the cell by binding to the ER. However, at premenopausal levels of estrogens, we show an association between the antiestrogenic effect and the levels of endoxifen corresponding to different CYP2D6 genotypes (41).

Estrogens activate the ER and induce its transcriptional activity via interaction with the estrogen-responsive gene promoters. Estrogen receptor protein turnover is required (42) to maintain the continuous transcription of mRNA. This turnover is achieved by proteosomal degradation of the ER protein. Binding of antiestrogens, in particular 4OHT, blocks the ER and promotes stabilization

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**Figure 5.** Effect of tamoxifen and metabolites on ERα protein stability and molecular model of 4-hydroxytamoxifen (4OHT) action. A) Protein levels of ERα in MCF-7 cells after 24-hour treatment with extensive metabolizer (EM) concentrations of tamoxifen metabolites with and without endoxifen. The levels of ERα protein after treatment with tamoxifen metabolites are similar to upregulation after treatment with 4OHT at EM concentration (Supplementary Table 1, available online). B) Protein levels of ERα in MCF-7 cells after treatment with 1nM E2, and tamoxifen primary metabolites 4OHT and endoxifen at 1µM individually and in combination. C) Endoxifen is docked within the same pocket that binds 4OHT in the antagonist conformation of ERα ligand binding domain. 4OHT = 4-hydroxytamoxifen.
of the ER protein (43). In contrast, Wu et al. (44) reported that endoxifen targets ERα protein for degradation in MCF-7 and T47D cells at higher concentrations (100 and 1000 nM) (44). However, these concentrations are not comparable with circulating endoxifen concentrations in patients. The same authors used (44) circulating EM and PM genotype concentrations of endoxifen with circulating concentrations of tamoxifen and its primary metabolites in MCF-7 cells and demonstrated ERα degradation only at EM levels of endoxifen. Endoxifen is biologically very similar to 4OHT in breast cancer cells (45). According to the molecular model of 4OHT action (Figure 5C) (34), the structurally similar endoxifen should bind to the ER and induce a similar conformation of the ER. The fact that the lone pair of electrons on the free nitrogen of endoxifen now interacts with the Asp351 means that it is not available to interact with the appropriate amino acid at Helix 12. This means that it is less likely to create a closed Helix 12, and the instability will result in destruction of accumulated ERα compared with 4OHT (Figure 5, A and B). This contrasts with the selective estrogen receptor modulator raloxifene and bazedoxifene that completely shield Asp351 and prevent Helix 12 closure. This is why the unstable complex is degraded (46,47). Fulvestrant, which completely disrupts the complex, is the extreme case of complex destruction (48).

Wu et al. (49) proposed a link between endoxifen-mediated destruction of ERα protein and levels of ERβ in the cells. However, the artificial overexpression of ERβ in cells may not accurately depict the natural mechanism of ERα degradation in tumors. MCF-7 cells have very low levels of ERβ expression compared with ERα. Breast tumors have very low expression of ERβ protein or mRNA (50–52) compared with ERα.

Tamoxifen and its metabolites regulate the transcriptional activity of estrogen-responsive genes. GREB1 plays a substantial role in breast cancer cell hormone-dependent proliferation (40). We demonstrate that tamoxifen and NDMTAM partially inhibit GREB1 estrogen-induced mRNA synthesis; however, addition of 4OHT and endoxifen statistically significantly enhances the antiestrogenic activity of the tamoxifen metabolite pool (Figure 4B). These results show that endoxifen, unlike the short-term cell growth end point, actually plays a substantial role in inhibiting the estrogen-mediated activation of responsive genes. We further examined the role of endoxifen in the estrogen regulation of pS2 and PgR gene expression (Figure 4, C and D). Although these genes are not important for proliferation, they illustrate the diversity of responsiveness to tamoxifen and its metabolites. The addition of tamoxifen and NDMTAM elevated both pS2 and PgR, an effect that has been noted previously (53,54). Adding the other tamoxifen metabolite 4OHT partially decreased gene expression of pS2 and PgR, but endoxifen suppressed gene expression further. It may be that these observations are relevant during prolonged adjuvant therapy (6) as incomplete suppression of gene function may lead to estrogen-stimulated proliferation, development of resistance, and, ultimately, recurrence. These conclusions are supported by a recent report by Hawse et al. (55).

This study also had some limitations. Although these data in vitro model the antiestrogen environment in postmenopausal patients taking tamoxifen in the short term, there are other dimensions to consider for therapeutic efficacy during adjuvant therapy. Tamoxifen and metabolites are competitive inhibitors of estrogen-induced cell proliferation (56). Therefore, circulating and tumor cell estrogen levels are critical for successful antiestrogen therapy. We have demonstrated (41) that the efficacy of the metabolite mix for tamoxifen, and the role of endoxifen, is critically dependent upon the circulating estrogen levels. The studies that titrate endoxifen in a parallel premenopausal model, ie, much higher circulating levels of estrone and estradiol, show that only by titrating down estrogen levels can the positive role of endoxifen be defined (41).

There is no laboratory model that can simulate the action of a decade of adjuvant therapy. It is now emerging that tamoxifen and metabolites are not simply acting just as inhibitors of estrogen action, but there is a dynamic change in breast cancer cell population over years as selection pressure creates acquired resistance in micrometastases. The principle has recently been illustrated by the extended cell culture of aromatase inhibitor-resistant breast cancer cells incubated in a cytostatic environment for eight weeks. Cell populations change dramatically (57). For the future, we propose to monitor the evolution of breast cancer cell populations under the selection pressure of appropriate antiestrogenic therapy for years. This will stimulate the evolution of cell populations during adjuvant therapy that become vulnerable to a woman’s own estrogen to trigger estrogen-induced apoptosis. This approach was originally used with tamoxifen to describe the evolution of acquired resistance of tumors in the athymic mouse model over years of treatment (58). This cytotrophic mechanism of tamoxifen and its metabolites needs time, compliance and high endoxifen levels to create effective selection pressure in different estrogen environments over years. It is these interlocking factors that expose the vulnerability of breast cancer to estrogen-induced apoptosis that results in a decrease in mortality in the decade after tamoxifen is stopped (59). However, to extrapolate appropriately to clinical care it is important to appreciate the requirement to validate methodologies to determine the CYP2D6 genotyping, as there is a profound loss of heterozygosity of the gene in breast cancer tissue. This quality control issue in genotyping was emphasized recently (60). In conclusion, based on the experimental data in vitro, in a panel of breast cancer cell lines in a postmenopausal setting, the presence of endoxifen in any concentration is secondary for blocking growth but necessary to block estrogen-regulated genes completely.

References


**Funding**

This work was supported by the Department of Defense Breast Program (award number W81XWH-06-1-0590) Center of Excellence, the Susan G. Komen for the Cure Foundation (award number SAC100009), and the Lombardi Comprehensive Cancer Center Support Grant (core grant NIH P30 CA051008). The views and opinions of the authors do not reflect those of the US Army or the Department of Defense.

**Affiliations of authors:** Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC (PYM, REM, DJF, PB, VRK, CJ); Institute of Chemistry, Romanian Academy, Timisoara, Romania (RFC).