

The Tamoxifen Metabolite, Endoxifen, Is a Potent Antiestrogen that Targets Estrogen Receptor α for Degradation in Breast Cancer Cells

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Abstract

Tamoxifen has been the most important therapeutic agent for the treatment of estrogen receptor (ER)-positive breast cancer for the past three decades. Tamoxifen is extensively metabolized by cytochrome P450 enzymes, and recent *in vivo* studies have shown that women with genetically impaired cytochrome P450 2D6 have reduced production of endoxifen and a higher risk of breast cancer recurrence. Despite these observations, the contribution of endoxifen to the overall drug effectiveness of tamoxifen remains uncertain. Here, we provide novel evidence that endoxifen is a potent antiestrogen that functions in part by targeting ER α for degradation by the proteasome in breast cancer cells. Additionally, we show that endoxifen blocks ER α transcriptional activity and inhibits estrogen-induced breast cancer cell proliferation even in the presence of tamoxifen, N-desmethyl-tamoxifen, and 4-hydroxytamoxifen. All of the effects of endoxifen are concentration dependent and do not occur at concentrations observed in human CYP2D6 poor metabolizers. These results support the theory that endoxifen is the primary metabolite responsible for the overall effectiveness of tamoxifen in the treatment of ER-positive breast cancer. [Cancer Res 2009;69(5):1722–7]

Introduction

Women diagnosed with estrogen receptor (ER)-positive breast cancer are commonly treated with tamoxifen. Tamoxifen is extensively metabolized by the cytochrome P450 enzyme system into 4-hydroxy tamoxifen (4HT), and N-desmethyl-tamoxifen (NDT; refs. 1, 2), followed by secondary metabolism to 4-hydroxy-N-desmethyl-tamoxifen (endoxifen; ref. 3). Although it has been assumed that 4HT is the primary means by which tamoxifen exerts its antibreast cancer effect, recent studies suggest that endoxifen is significantly more potent than tamoxifen and equipotent to 4HT in its ability to bind to ER α and ER β , and in suppression of ER-dependent breast cancer proliferation (4, 5).

Endoxifen is formed in the liver by the CYP2D6-mediated oxidation of NDT (1, 3, 6) and CYP2D6 polymorphisms and drug-induced inhibition of CYP2D6 enzyme activity significantly reduce endoxifen concentrations in humans (3, 7, 8). Retrospective analyses of four separate cohorts of women receiving adjuvant tamoxifen showed that women with impaired CYP2D6 metabolism have a significantly higher risk of breast cancer recurrence (9–13),

and while taking tamoxifen, are less likely to report hot flashes (10). Although the clinical observations suggest that endoxifen may be an important tamoxifen metabolite, little is known about the actions of endoxifen on the ER or the mechanism by which it functions to suppress breast cancer progression. Furthermore, some have speculated that the ER is fully saturated by tamoxifen (14), which would diminish the importance of variability in endoxifen concentrations. Therefore, we sought to further characterize the ability of endoxifen to block ER function and repress breast cancer cell proliferation, and to determine whether the effects of endoxifen on breast cancer cells are maintained in the presence of tamoxifen and its primary metabolites.

Materials and Methods

Cell culture and chemicals. MCF7, T47D, and parental Hs578T cells were purchased from American Type Culture Collection. Ishikawa cells were purchased from Sigma-Aldrich. These cell lines were grown in phenol red-free DMEM/F12 medium containing 10% (v/v) fetal bovine serum (FBS). Hs578T-ER α and U2OS-ER α cells were developed and cultured as previously described (15, 16). All cell treatments were performed in DMEM/F12 medium containing 10% triple charcoal stripped FBS. (Z)-endoxifen was synthesized by Dr. Abdul Fauq (Mayo Clinic, Jacksonville, FL). All ER ligands used in this study were resuspended in 100% ethanol.

Cell treatments. For analysis of ER α protein levels after ER ligand treatments, cells were plated in 12-well tissue culture plates and treated as indicated. Doxycycline was included in the cell culture medium at a final concentration of 100 ng/mL throughout all experiments involving the Hs578T-ER α or U2OS-ER α cells to induce ER α expression. For MG132 treatments, cells were plated as above and pretreated with either DMSO vehicle or 25 μ mol/L MG132 for 1 h. Cells were then treated for an additional 8 h with indicated ER ligands in the presence of either DMSO or MG132.

Protein isolation and Western blotting. Following ER ligand treatments, cells were washed twice with 1 \times PBS and cell extracts were prepared using Laemmli buffer (Bio-Rad). ER α protein levels were analyzed in the MCF7, T47D, and Ishikawa cells using an anti-ER α antibody (HC-20). A flag-specific antibody (Sigma-Aldrich) was used to detect ER α protein in the Hs578T-ER α and U2OS-ER α cell lines. Tubulin protein levels were measured using an anti-tubulin antibody (Sigma Aldrich). Protein levels were visualized using enhanced chemiluminescence (Amersham Biosciences).

Transient transfection and luciferase assays. Parental Hs578T cells were plated in 12-well tissue culture plates and transfected in triplicate with 250 ng per well of the ERE-TK-luciferase reporter construct and 250 ng per well of an ER α expression construct (PCDNA4/TO Flag ER α) using Fugene 6 (Roche Applied Science). After transfection, cells were treated as indicated for 24 h. Cells were lysed and equal amounts of extract were assayed for luciferase activity.

Real-time reverse transcriptase PCR. MCF7 cells were plated in 100-mm tissue-culture plates and treated as indicated for 24 h. Total RNA was isolated using Trizol reagent (Invitrogen). Five hundred nanograms of total RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was performed as described previously (17). Primers specific for amphiregulin and c-Myc were as follows: amphiregulin

Note: X. Wu and J.R. Hawse contributed equally to this work.

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forward, 5'-GGGAGTGAGATTTCCCTGT-3' and amphiregulin reverse, 5'-AGCCAGGTATTGTGGTTCG-3'; c-Myc forward, 5'-GCCACGTCTCCACACATCAG-3' and c-Myc reverse, 5'-TCTTGGCAGCAGGATAGTCCT-3'. Control primers specific for human TATA binding protein were as follows: forward, 5'-AGTTGTACAGAAGTTGGGTTTC-3' and reverse, 5'-AACAAATCTGGGT-TGATCATTC-3'.

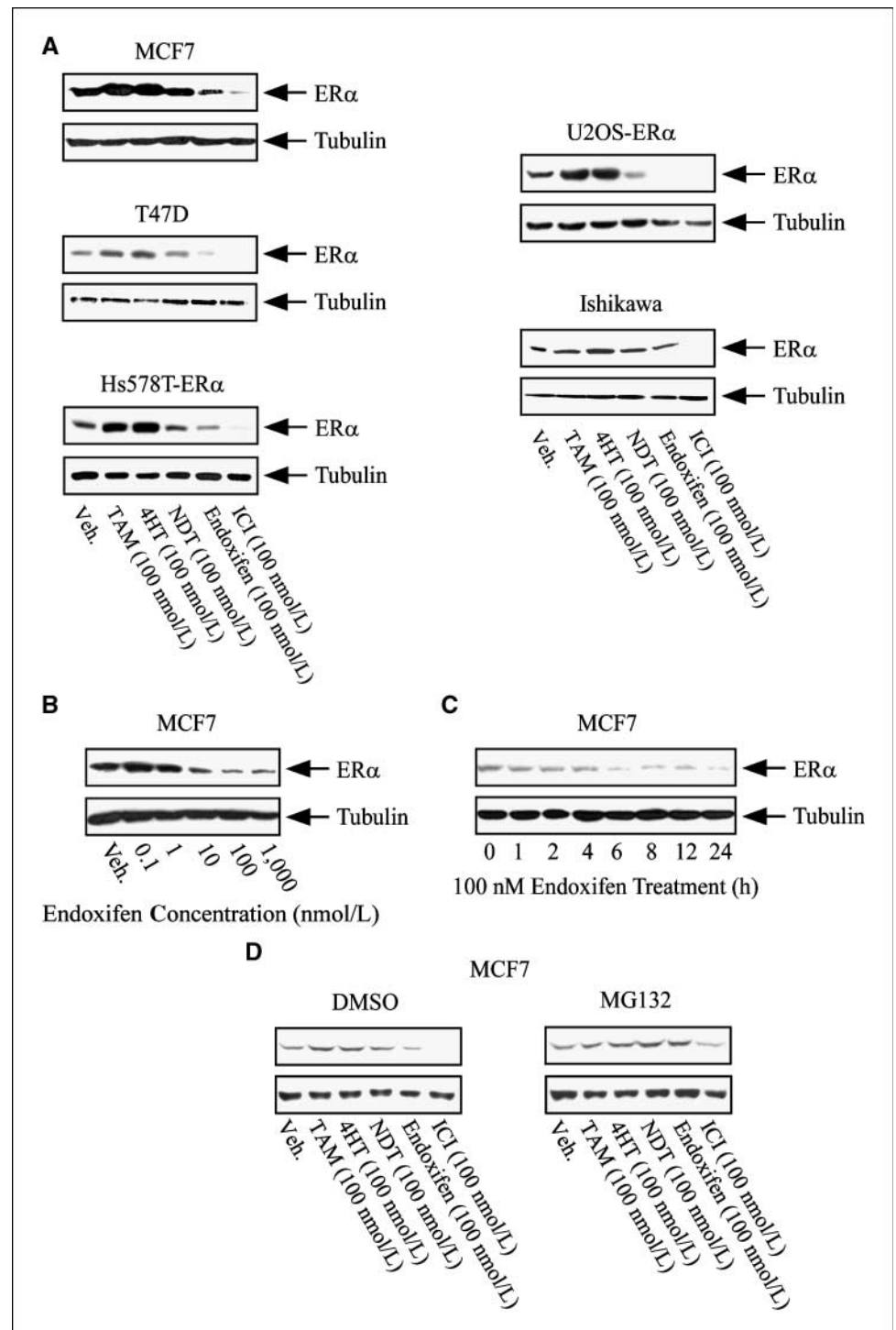
Cell proliferation assays. MCF7 and Ishikawa cells were grown in 10% triple charcoal-stripped serum-containing medium for 3 d. The cells were then plated at a density of 2,000 cells per well in 96-well tissue culture plates and treated as indicated every 48 h. Cell proliferation assays were conducted 8 days after the first treatment using a CellTiter-Glo

Luminescent Cell Viability kit (Promega) according to the manufacturer's protocol.

Results and Discussion

We first sought to determine if endoxifen had actions similar to that of 4HT or ICI at the level of ER stability/degradation. Treatment of MCF7 cells, which endogenously express ER α , with tamoxifen and 4HT results in stabilization of the ER (Fig. 1A). However, treatment with endoxifen reduces ER α protein levels

Figure 1. ER α protein levels in response to endoxifen treatment. **A**, Western blot analysis of ER α protein levels in MCF7, T47D, Hs578T-ER α , U2OS-ER α , and Ishikawa cells treated as indicated for 24 h. Veh, vehicle; TAM, tamoxifen. **B**, Western blot analysis of ER α protein levels in MCF7 cells treated with various concentrations of endoxifen for 24 h. **C**, Western blot analysis of ER α protein levels in MCF7 cells treated with endoxifen (100 nmol/L) for indicated times. **D**, Western blot analysis of ER α protein levels in MCF7 cells exposed to DMSO vehicle or 25 μ mol/L MG132 and treated as indicated for 8 h. Tubulin levels are shown as protein loading controls.



similar to that of ICI (Fig. 1A). No significant changes were observed in ER α protein levels after treatment with NDT (Fig. 1A). Similar results were also observed in T47D cells (Fig. 1A). These observations were further confirmed in Hs578T breast cancer cells stably expressing ER α (Fig. 1A; ref. 15). The same patterns of ER α protein levels were observed in U2OS-ER α osteosarcoma cells (Fig. 1A). However, endoxifen treatment did not result in reduced ER α protein levels in Ishikawa endometrial carcinoma cells (Fig. 1A).

To determine the concentration of endoxifen that optimally increases ER α protein turnover, dose response studies in MCF7 cells were performed. These studies revealed that endoxifen concentrations between 10 and 1,000 nmol/L enhance ER α protein degradation (Fig. 1B). Lower concentrations of endoxifen (≤ 1 nmol/L) do not affect ER α protein levels (Fig. 1B). We next performed a time course study using 100 nmol/L endoxifen concentrations to analyze the rate at which ER protein turnover occurs. These studies revealed significant decreases in ER α protein levels within 6 hours of endoxifen treatment (Fig. 1C). To show that the decreased ER α protein levels were due to protein degradation, the proteasome was inhibited with MG132. Inhibition of proteasome activity blocks ER α degradation in response to endoxifen (Fig. 1D). These data show that endoxifen functions differently from its parent compound, tamoxifen, and the other tamoxifen metabolites, with regard to its effect on ER α protein stability.

The above studies describe the individual effects of tamoxifen, 4HT, NDT, and endoxifen on ER α stability. However, in a patient receiving tamoxifen therapy, these metabolites are present together at various concentrations. In women taking tamoxifen at a dose of 20 mg/day, the average steady-state concentrations of tamoxifen, 4HT, and NDT are 335, 7.4, and 695 nmol/L, respectively (7, 8). Conversely, the plasma concentrations of endoxifen vary widely and are dependent on the activity of CYP2D6 enzyme. The CYP2D6 phenotypes which result from common genetic polymorphisms include poor (PM), intermediate (IM), extensive (EM), and ultrarapid (UM) metabolizers. Extensive metabolizers typically have plasma endoxifen concentrations of 90 nmol/L (± 40 nmol/L), intermediate metabolizers between 40 and 60 nmol/L, and poor metabolizers < 30 nmol/L (7).

Because endoxifen is the only tamoxifen metabolite that elicits increased ER α protein degradation in breast cancer cells, we hypothesized that high endoxifen concentrations corresponding to the levels seen in CYP2D6 EM would result in increased ER α protein turnover even in the presence of tamoxifen and the other tamoxifen metabolites, whereas low concentrations of endoxifen (corresponding to CYP2D6 PM) would not. To determine if endoxifen could out-compete tamoxifen, 4HT, and NDT, we pretreated MCF7 cells for 4 hours using the average concentration of these metabolites that exist in patients receiving the standard dose of tamoxifen therapy (20 mg/day). This was followed by endoxifen treatments of 1,000, 100, 40, or 20 nmol/L for an additional 20 hours to mimic the various CYP2D6 phenotypes. As expected, treatment of MCF7 cells with a combination of tamoxifen, 4HT, and NDT resulted in ER α protein stabilization (Fig. 2). Treatment of cells with high concentrations of endoxifen (100–1,000 nmol/L) resulted in significant decreases in ER α protein levels, even in the presence of tamoxifen and the other metabolites (Fig. 2). In contrast, low concentrations of endoxifen (20–40 nmol/L) did not induce ER α protein degradation (Fig. 2). These results were confirmed in Hs578T and U2OS cells stably expressing ER α (Fig. 2). These data show that the concentrations of

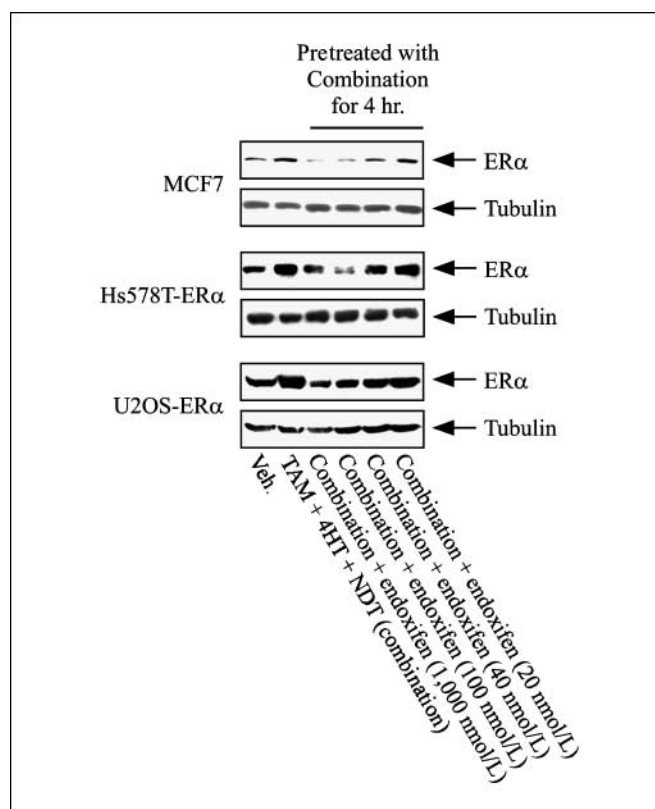


Figure 2. High concentrations of endoxifen (100–1,000 nmol/L) compete with tamoxifen (300 nmol/L), 4-hydroxytamoxifen (7 nmol/L), and NDT (700 nmol/L), resulting in increased ER α protein degradation, whereas low concentrations of endoxifen (20–40 nmol/L) do not. Western blot depicting ER α protein levels in various cell lines treated as indicated for a total of 24 h. Tubulin levels are shown as protein loading controls.

endoxifen typically seen in CYP2D6 EM are able to induce ER α degradation, even in the presence of tamoxifen and the other tamoxifen metabolites.

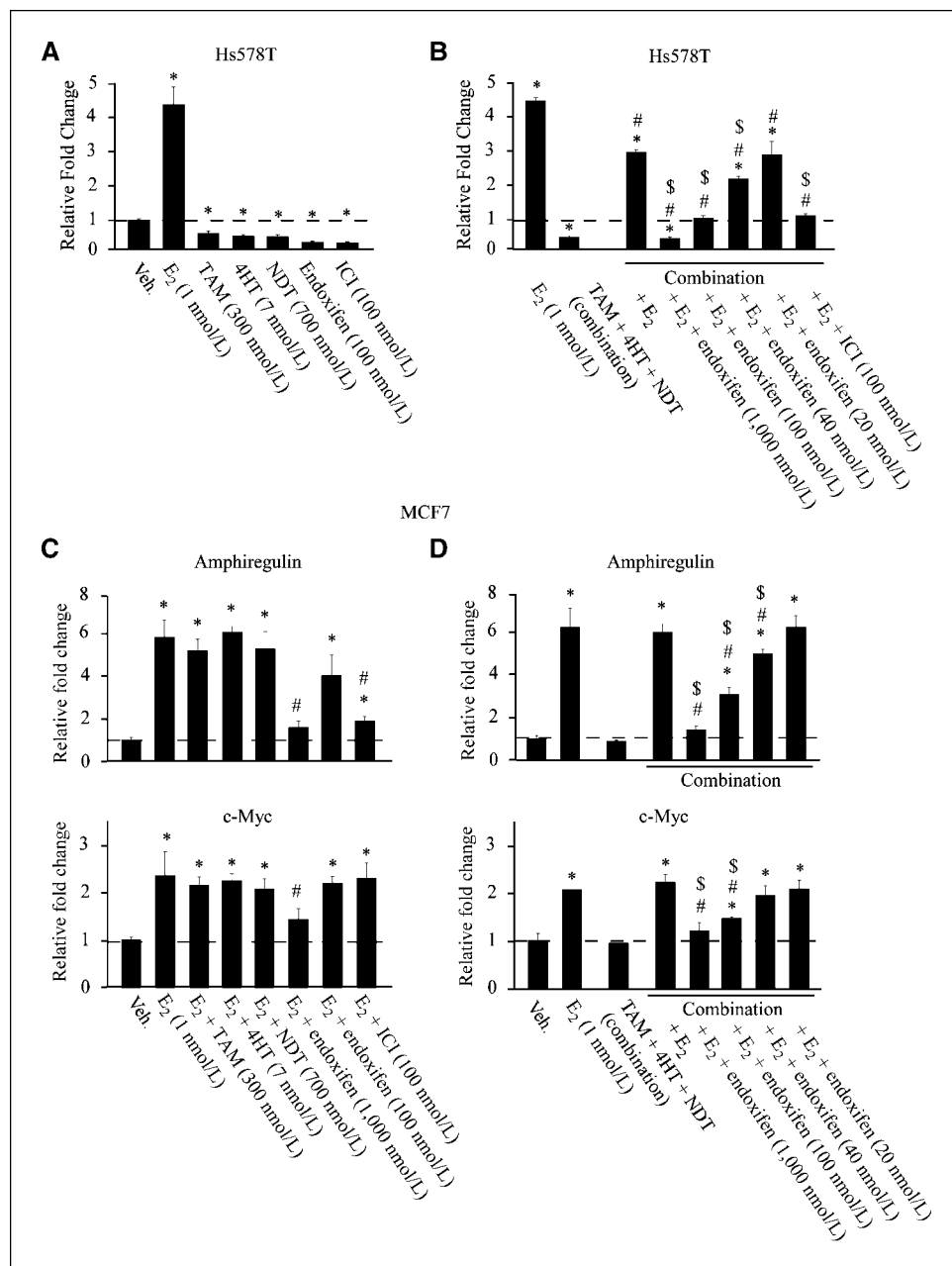
To determine whether or not endoxifen blocks ER α function, we examined the transcriptional activation of an estrogen response element (ERE). Parental Hs578T cells were transiently transfected with an ER α expression construct and an ERE-luciferase reporter construct and treated as indicated for 24 hours. The results of these studies show that the ERE is activated in the presence of estrogen and is repressed to varying degrees by all of the tamoxifen metabolites as well as ICI (Fig. 3A). Next, we sought to determine if a combination of tamoxifen, 4HT, and NDT could block estrogen-induced ERE activity. As shown in Fig. 3B, a combination of these three selective estrogen receptor modulators slightly reduces ERE activity in the presence of estrogen. However, the addition of high concentrations of endoxifen (100–1,000 nmol/L) completely block ERE activation, whereas low concentrations (20–40 nmol/L) are less effective (Fig. 3B). The same patterns of ERE activation and repression were observed when using 10 nmol/L concentrations of estrogen (data not shown).

To confirm these results and to determine if endoxifen is able to block the estrogen activation of endogenous genes, we analyzed the expression levels of amphiregulin and c-Myc in MCF7 cells. Amphiregulin and c-Myc were chosen based on the fact that they are well known targets of ER α that play important roles in breast cancer. As shown in Fig. 3C, amphiregulin and c-Myc expression are induced by estrogen treatment. Concentrations of tamoxifen,

4HT, and NDT observed in patients receiving tamoxifen therapy do not block estrogen activation of these genes; however, high concentrations of endoxifen either completely block or significantly reduce estrogen activation (Fig. 3C). Additionally, the combination of tamoxifen, 4HT, and NDT are unable to block the estrogen activation of amphiregulin and c-Myc (Fig. 3D). However, the addition of high concentrations of endoxifen either completely block or significantly reduce this activation, whereas low concentrations of endoxifen do not (Fig. 3D). The same patterns of amphiregulin and c-Myc activation and repression were observed when using 10 nmol/L concentrations of estrogen (data not shown). These results are in agreement with the ERE studies (Fig. 3A and B) and again show that endoxifen, at concentrations observed in patients, is the most potent tamoxifen metabolite with regard to its ability to suppress ER α -mediated transcription.

It is well-accepted that estrogen induces ER-positive breast cancer cell proliferation both *in vivo* and *in vitro*, and previous studies have clearly shown that endoxifen is equipotent with 4HT in inhibiting breast cancer cell proliferation (4, 5). However, we sought to confirm whether endoxifen could inhibit estrogen-induced proliferation even in the setting of tamoxifen, 4HT, and NDT. As indicated in Fig. 4, treatment of MCF7 cells with estrogen results in a significant increase in their proliferation rate. The combination of tamoxifen, 4HT, and NDT modestly reduces the rate of MCF7 cell proliferation in response to estrogen (Fig. 4). Interestingly, a dose response is observed with the addition of endoxifen. Although low concentrations of endoxifen (20–40 nmol/L) significantly repress the estrogen-induced growth of MCF7 cells, high endoxifen concentrations (100–1,000 nmol/L) either completely block or drastically repress this response (Fig. 4). As with the

Figure 3. ER α transcriptional activity is blocked by endoxifen in Hs578T and MCF7 cells. *A* and *B*, an ER α expression vector and a consensus ERE-reporter construct (luciferase) were transiently transfected into parental Hs578T cells and treated as indicated for 24 h. Luciferase values are reported as relative fold change compared with ethanol (vehicle). *, significance at the $P < 0.05$ level (ANOVA) compared with vehicle. #, significance at the $P < 0.05$ level (ANOVA) compared with estrogen treatment. \$, significance at the $P < 0.05$ level (ANOVA) compared with a combination of tamoxifen (300 nmol/L), 4-Hydroxytamoxifen (7 nmol/L), and NDT (700 nmol/L) plus estrogen (1 nmol/L). *C* and *D*, real-time reverse transcription-PCR analysis of amphiregulin and c-Myc gene expression in MCF7 cells after indicated treatments for 24 h. *, significance at the $P < 0.05$ level (ANOVA) compared with vehicle. #, significance at the $P < 0.05$ level (ANOVA) compared with estrogen treatment. \$, significance at the $P < 0.05$ level (ANOVA) compared with a combination of tamoxifen (300 nmol/L), 4-hydroxytamoxifen (7 nmol/L), and NDT (700 nmol/L) plus estrogen (1 nmol/L).



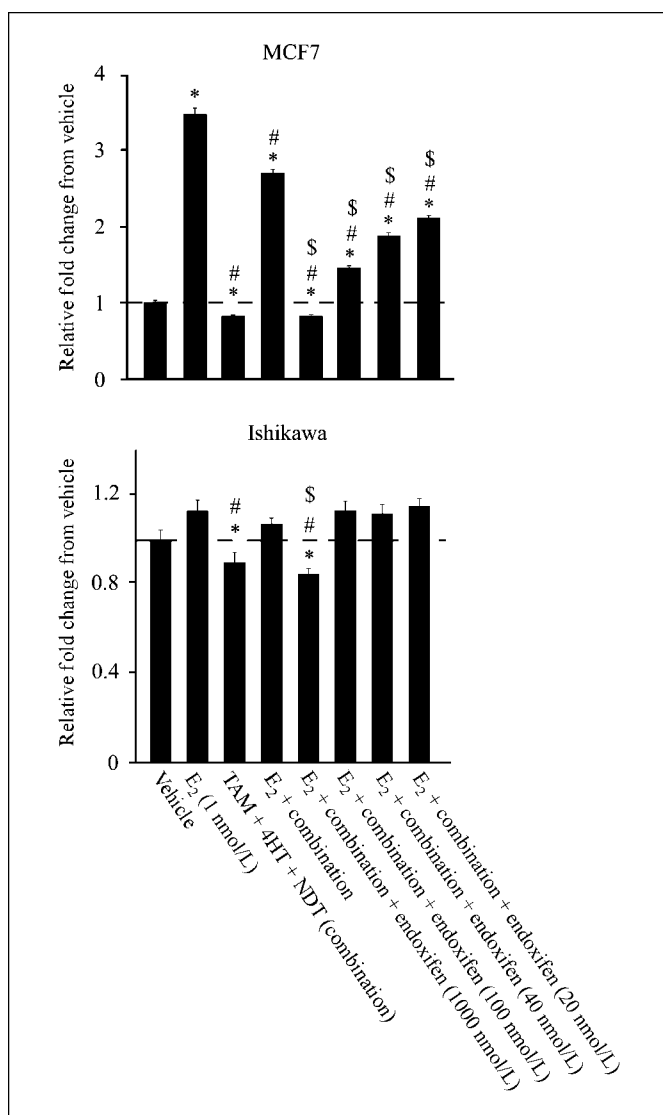


Figure 4. Effects of endoxifen on MCF7 and Ishikawa cell proliferation. MCF7 and Ishikawa cells were treated as indicated for 8 d and cell proliferation rates were analyzed. Graphs depict fold change from vehicle-treated cells. *, significance at the $P < 0.05$ level (ANOVA) compared with vehicle control. #, significance at the $P < 0.05$ level (ANOVA) compared with estrogen-treated cells (10 nmol/L). \$, significance at the $P < 0.05$ level (ANOVA) compared with a combination of tamoxifen (300 nmol/L), 4-hydroxytamoxifen (7 nmol/L), and N-desmethyl tamoxifen (700 nmol/L) plus estrogen (1 nmol/L).

gene expression studies, the same patterns of MCF7 cell proliferation were observed when using 10 nmol/L concentrations of estrogen (data not shown). These results are in agreement with the transcriptional studies described in Fig. 3 and again show that endoxifen is the most potent tamoxifen metabolite at concentrations found in patients receiving tamoxifen therapy. To determine if endoxifen behaves similarly in endometrial cancers, Ishikawa cells were treated in the same manner as above. Although

estrogen treatment of these cells did not result in a significant increase in their proliferation, this is likely due to the fact that 1 nmol/L estrogen concentrations were used. Indeed, others have reported increased proliferation rates of Ishikawa cells when much higher concentrations of estrogen are used (18, 19). The addition of 1,000 nmol/L endoxifen resulted in a slight but significant decrease in cell proliferation in the presence of 1 nmol/L estrogen and a combination of tamoxifen, 4HT, and NDT. However, lower concentrations of endoxifen (20–100 nmol/L) had no effect on the proliferation rates of these cells relative to estrogen alone or to the combination treatment of estrogen plus tamoxifen, 4HT, and NDT. These data suggest that endoxifen is relatively ineffective at either inducing or repressing Ishikawa cell growth under conditions mimicking that of a patient receiving the standard dose of tamoxifen therapy.

In this article, we show that endoxifen, a tamoxifen metabolite, is a potent antiestrogen that mimics the actions of ICI with regard to its ability to target ER α for degradation by the proteasome, to block ER α -mediated transcriptional activation and to inhibit estrogen-induced breast cancer cell proliferation. It is important to note that these effects of endoxifen are maintained even in the presence of tamoxifen, 4HT, and NDT at concentrations observed in patients. Of particular importance, concentrations of endoxifen mimicking those found in the plasma of CYP2D6 EM are much more potent than those of a CYP2D6 PM. These data correlate well with the clinical observations demonstrating CYP2D6 genotype as a predictive marker for the effectiveness of tamoxifen. To our knowledge, this is the first study to show that the mechanism of action of endoxifen is different than that of 4HT because it targets the ER for degradation. Additionally, these data suggest that the optimal drug effect of tamoxifen is directly related to endoxifen concentrations and the ability of this metabolite to degrade rather than stabilize the ER. Furthermore, these studies confirm and provide a basis for CYP2D6 genotyping as a means of individualizing the optimal endocrine therapy for women with endocrine-responsive breast cancer. Finally, this study lays the ground work for developing endoxifen, or related compounds, as an alternative drug treatment for women with ER-positive breast tumors.

Disclosure of Potential Conflicts of Interest

M.P. Goetz and J.N. Ingle have been consultants for Roche regarding CYP2D6 and tamoxifen and have a patent pending. The other authors declare no potential conflict of interest.

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