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ORIGINAL ARTICLE

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Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen

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Abstract *Purpose*: Tamoxifen is an effective drug for the treatment and prevention of breast cancer. It is extensively metabolized by the human cytochrome P450 enzyme system into several metabolites. Of these, 4-hydroxy-tamoxifen (4-OH-Tam) is an active metabolite, which has greater anti-estrogenic potency than the parent drug, tamoxifen. We reported recently that 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) could also be active. The progesterone receptor (PR) messenger ribonucleic acid (mRNA) expression is commonly studied as a marker of estrogenic effect in breast cancer cells and PR levels in breast cancer patients are correlated with tamoxifen response. We, therefore, determined the effect of endoxifen and 4-OH-Tam on 17β-estradiol (E2)-induced PR mRNA expression in an estrogen receptor-positive human breast cancer cell line. *Methods*: MCF-7 cells were treated with drugs for 24 h. The total ribonucleic acid (RNA) was harvested and transcribed into complementary deoxyribonucleic acids (cDNAs). The PR mRNA level was measured by using real-time reverse transcription polymerase chain reaction (RT-PCR). The PR expression data were normalized using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. We measured the metabolite concentrations in the cultured media by high performance liquid chromatography (HPLC) to determine whether there was conversion of one metabolite to the other. *Results*: Consistent with previous reports, the dose–response of the E2 effect on the PR expression indicated an ED_{50} value of approximately 60 pM and the maximum induction of PR mRNA was nearly ten-fold. When 10^{-10} M E2 was used, induction of the PR expression was observed in 2 h and reached its maximum at 24 h. In this assay, neither endoxifen nor 4-OH-Tam alone produced any change in the PR mRNA expression. However, both endoxifen and 4-OH-Tam decreased the E2-induced PR expression with similar potency. There was very little interconversion between the two metabolites during the culture. *Conclusions*: Since endoxifen is present at greater concentrations than 4-OH-Tam in human plasma of breast cancer patients receiving chronic tamoxifen, these results provide further evidence that endoxifen is as important as, or more important than, 4-OH-Tam to the anti-estrogenic action of tamoxifen.

Keywords Breast cancer · Tamoxifen · 4-hydroxy-tamoxifen · Endoxifen · Progesterone receptor · mRNA expression

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Introduction

Tamoxifen is a selective estrogen receptor modulator (SERM) that is widely used in the treatment and prevention of breast cancer [9, 13, 18, 35]. However, there is substantial interpatient variability in both the development of resistance to tamoxifen therapy [39] and the occurrence of its adverse reactions, such as hot flushes, deep vein thrombosis (DVT), and the occurrence of endometrial cancer [3, 16, 32]. The mechanisms causing this variability are poorly understood. Tamoxifen is extensively metabolized by the cytochrome P450 enzyme system in vivo into several metabolites that have variable potencies towards the estrogen receptors. Tamoxifen has been considered as a prodrug that has increased activity after activation in vivo. Previous studies have identified

the major tamoxifen metabolites to be N-desmethyl-tamoxifen (NDM), 4-hydroxy-tamoxifen (4-OH-Tam), tamoxifen-N-oxide, α-hydroxy-tamoxifen, and N-didesmethyl-tamoxifen [8, 11, 27, 30, 40]. Among these, 4-OH-Tam has been shown to be a potent anti-estrogen (30–100-fold more potent than tamoxifen itself) [7, 22]. For this reason, some investigators have considered 4-OH-Tam to be the active metabolite of tamoxifen and, thus, it is frequently used to characterize tamoxifen activity in vitro.

Work by Lien et al. [28] has identified 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) in the human bile of one breast cancer patient receiving chronic tamoxifen treatment, although the activities of this metabolite have not been studied. Recently, we have demonstrated that 4-hydroxy-N-desmethyl-tamoxifen, is equipotent to 4-OH-Tam in estrogen receptor-alpha (ERα) and receptor-beta (ERβ) binding, as well as in the inhibition of 17β-estradiol (E2)-induced proliferation in human breast cancer cells [20, 40]. This metabolite is likely to be more important clinically than 4-OH-Tam because we also have pharmacokinetic data indicating that the average plasma endoxifen concentrations are higher (>six-fold) than 4-OH-Tam in breast cancer patients who are taking tamoxifen chronically [27, 40]. Furthermore, we reported that the plasma concentrations of endoxifen are highly variable, and that the variability is due to polymorphisms in the CYP2D6 gene, the major metabolizing enzyme that forms endoxifen from NDM, and to drug interactions with CYP2D6 inhibitors [11, 40]. Therefore, it is important to understand the biological effects of endoxifen in assays that are frequently used to assess the anti-estrogenicity of SERMs.

One of the bioassays that are most frequently used to test the estrogenic/anti-estrogenic activity of SERMs and other estrogens is the induction or repression of progesterone receptor (PR) expression in breast cancer cells. The PR is an important gene that is primarily regulated by estrogens in vitro [6, 12, 31] and is known to be an important determinant of the effectiveness of tamoxifen therapy in vivo [2, 5, 26, 36]. Therefore, the studies described here were specifically designed to determine the effect of endoxifen on the PR expression and compare its potency to that of 4-OH-Tam.

Materials and methods

Drugs

E2, pure Z (trans)-isomers (>98% Z) of 4-OH-Tam, and a mixed form of 70% Z-isomers and 30% E (cis)-isomers of 4-OH-Tam were purchased from Sigma (St Louis, Missouri, USA). Endoxifen (mixture of 75% Z-isomers and 25% E-isomers) was synthesized by Dr. Ross Weatherman in the Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, Indiana, USA and its synthetic method is described in our previous report [20]. All drugs were dissolved in 0.1% ethanol, which does not produce any effect on the PR expression in MCF-7 cells.

Cell culture and drug treatment

Estrogen receptor-positive human breast carcinoma cell line (MCF-7 cells) were maintained at 37°C under a humidified atmosphere of 5% CO_2 and 95% air in an improved minimum essential media (IMEM) supplemented with 10% fetal bovine serum. Before the experimental treatments, these cells were preconditioned in charcoal-stripped calf serum for 3 days to remove the estrogens from the growth medium containing fetal bovine serum. The cells were treated with vehicle (0.1% ethanol), various doses of E2, 4-OH-Tam, endoxifen, or combinations for 24 h, in IMEM medium supplemented with 10% charcoal-stripped calf serum.

Ribonucleic acid (RNA) isolation

The total ribonucleic acid (RNA) from cells treated with drugs for 24 h was harvested for PR messenger ribonucleic acid (mRNA) analysis. RNA was extracted from approximately 2.5×10^5 cells by RNeasy Mini Kit (Qiagen Inc., Valencia, California, USA). DNase treatment was done by using DNA-free (Ambion Inc., Austin, Texas) in order to remove the remnant deoxyribonucleic acid (DNA) in the extracted total RNA. The RNA concentration was measured by A260, and the quality of the RNA was assessed by visualizing the 28S and 18S ribosomal RNA on the Agilent RNA 6000 Nano LabChip Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The RNA was stored at −80°C until used.

Complementary deoxyribonucleic acid (cDNA) synthesis

Complementary deoxyribonucleic acid (cDNA) for the real-time quantitative polymerase chain reaction (PCR) assay was synthesized from DNase-treated total RNA using the Promega Reverse Transcription System (Promega Corporation, Madison, Wisconsin, USA).

Real-time quantitative polymerase chain reaction (PCR) (TaqMan) assay for PR mRNA

The cDNA was amplified with the Platinum Quantitative PCR Supermix-UDG (Invitrogen Corporation, Carlsbad, California, USA) using cDNA equivalent to 50 ng of total RNA in the iCycler real-time PCR machine (Bio-Rad Laboratories, California, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was quantified to normalize each sample. The PR and GAPDH reagents were multiplexed in the same

tube. The progesterone receptor assay conditions were kindly provided by Gregory L. Shipley (University of Texas Medical School, Houston, Texas, USA). The sequences of primers and probe for the PR gene were as follows: forward primer, 5′-GAG CAC TGG ATG CTG TTG CT-3′; reverse primer, 5′-GGC TTA GGG CTT GGC TTT C-3′; probe, 5′-FAM-TCC CAC AGC CAT TGG GCG TTC-BHQ1-3′, GAPDH primers and probe, forward primer, 5′-GAA GGT GAA GGT CGG AGT C-3′; reverse primer, 5′-GAA GAT GGT GAT GGG ATT TC-3′; probe, 5′-TxRed-CAA GCT TCC CGT TCT CAG CC-BHQ2-3′. The sequence of single-stranded PR DNA used for obtaining the standard curve of PR quantitative reverse transcription polymerase chain reaction (RT-PCR) was 5′-GAG CAC TGG ATG CTG TTG CTC TCC CAC AGC CAT TGG GCG TTC CAA ATG AAA GCC AAG CCC TAA GCC-3′. The thermal cycling conditions were composed of an initial step of UDG carry-over decontamination (one cycle of 50°C for 2 min and 95°C for 2 min), and then 42 cycles at 95°C for 15 s and 60°C for 1 min. Quantitative values of amplification were obtained from the threshold cycle (*Ct*) defined as the cycle number at which the fluorescent signal is first recorded above the background and is determined during the exponential phase of PCR rather than at the endpoint. In every PR assay, the standard curve of the PR single-stranded DNA showed good linear correlation for the wide range of dilutions and the slope approaches a theoretical doubling value of 3.3. The data expressed as the absolute PR mRNA value was similar to that expressed as normalized ratios (PR *Ct* over GAPDH *Ct*), therefore, only the absolute values are presented.

Measurement of tamoxifen metabolites in culture media

A 10-ml sample of culture media was collected in a plain glass tube. The concentrations of both Z-isomers and E-isomers of 4-OH-Tam and endoxifen in the culture media were determined using high performance liquid chromatography (HPLC), as described in detail in our earlier publication [11]. Briefly, on-line derivatization with minor modifications [14, 27] was used and this method allows the rapid extraction and specific detection of tamoxifen and its metabolites based on a technique originally described by Kikuta and Schmid [24], but employing a novel column switching technique [14] that allows for higher recovery and more precise separation.

Results

Dose–response relationship of E2-induced PR mRNA expression

MCF-7 cells in different laboratories, and even within the same laboratories, are known to have variable sensitivities to estrogens and anti-estrogens. Therefore, in order to conduct a detailed analysis of the effects of endoxifen on PR expression levels, it was necessary to establish the optimal assay conditions for the specific cells used in our laboratory. We present those data in Fig. 1. The MCF-7 cells were treated with E2 (10^{-13}–10^{-7} M) for 24 h, the total RNA was extracted and transcribed into cDNA, and PR mRNA was measured by real-time quantitative RT-PCR. As shown in Fig. 1, with E2-induced PR expression in the range of 10^{-11}–10^{-9} M. Concentrations above 10^{-9} M had no additional affect. The concentration that produces 50% of maximal response (EC_{50}) was calculated as 6×10^{-11} M (60 pM), which is similar to the results previously reported [1]. The maximum induction of PR mRNA expression was shown to be nearly tenfold (data not shown). Based on these results, we chose 10^{-10} M (100 pM) E2 for the tamoxifen antagonism studies to allow detection of both agonist and antagonist activities.

Time-course experiment of E2-induced PR mRNA expression

We determined the most appropriate time point to use in the bioassay by conducting a time-course experiment of E2 on the PR expression (Fig. 2). PR mRNA was measured at 0 h, 0.5 h, 1 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h after treatment with E2 (10^{-10} M). Induction of the PR expression was first observed at 2 h after E2 treatment and was maximal by 24 h. The expression level of PR mRNA at 48 h and 72 h after the treatment

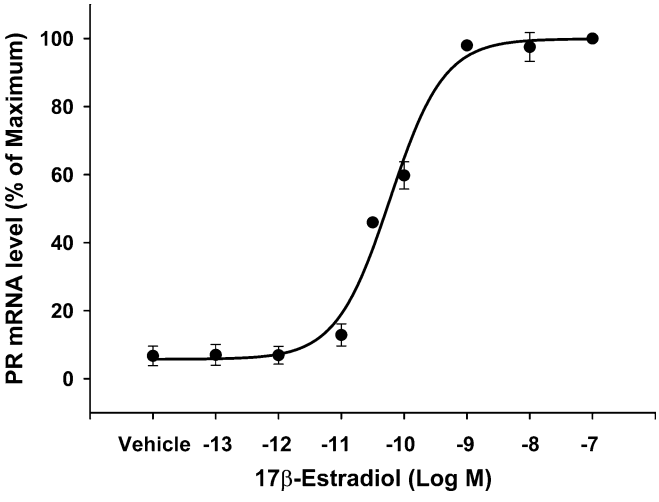


Fig. 1 Dose–response curve of E2-induced PR mRNA expression in ER-positive MCF-7 breast carcinoma cell line. Total RNA was extracted after 24 h treatment and was transcribed into cDNA, from which PR mRNA was measured by real-time quantitative RT-PCR. The vehicle was 0.1% ethanol. The EC_{50} value (the dose that produces half-maximal response) of E2-induced PR expression was 6×10^{-11} M (60 pM). An E2 dose of 10^{-10} M was chosen for further experiment to determine the effects of tamoxifen metabolites on PR expression. Mean values and SE of two experiments are shown

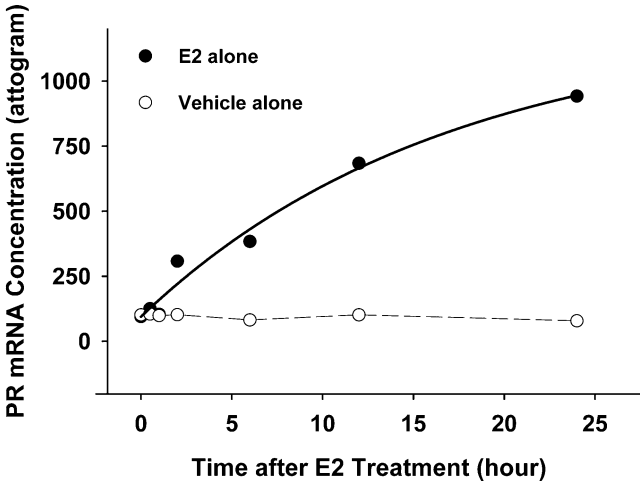


Fig. 2 Time-course experiment of induction of PR mRNA expression by 10^{-10} M E2 in MCF-7 cells. Level of PR mRNA expression was measured at 0 h, 0.5 h, 1 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h after administration of E2 (10^{-10} M). Ethanol (0.1%) was used as the vehicle. The time point of 24 h was selected for subsequent experiments to investigate the effects of tamoxifen metabolites on PR expression in these cells

was similar to that at 24 h (data not shown). The PR expression did not change in the vehicle-treated cells, as expected. Therefore, the 24-h time point was selected as the appropriate time point for testing the effect of the tamoxifen metabolites on the PR expression in these cells.

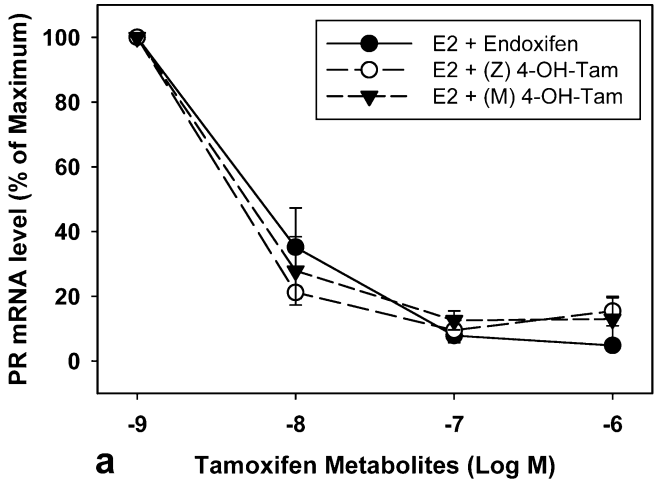
Effect of 4-OH-Tam and endoxifen on PR mRNA expression

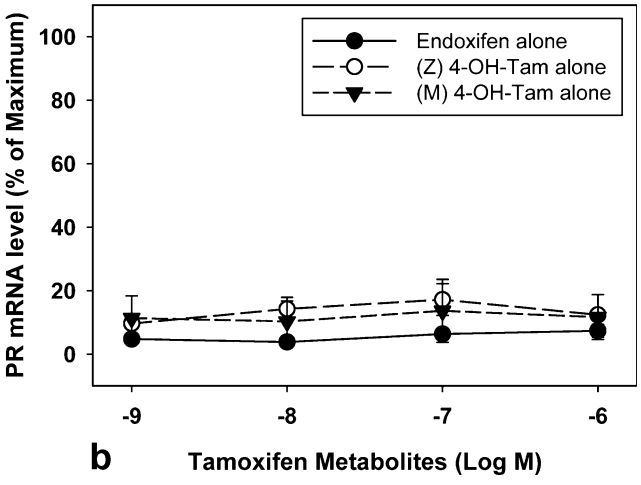
Since the 4-OH-Tam that was available was either 70:30 Z:E or pure Z, we tested these two preparations of 4-OH-Tam to determine if there was any difference in the efficacy of the isoforms. The MCF-7 cells were treated with the two 4-OH-Tam isoforms in the presence (Fig. 3a) or absence (Fig. 3b) of E2 (10^{-10} M). The dose–dependent antagonizing effects of both forms of 4-OH-Tam on the E2-induced PR expression were observed in the range 10^{-9}–10^{-6} M of 4-OH-Tam. Maximal effects were observed with 10^{-7} M, which almost completely blocked the E2-induced PR expression in these cells (Fig. 3a). In the absence of E2, neither preparation affected the PR mRNA expression (Fig. 3b). The effective concentration of 4-OH-Tam, 10^{-8} M (10 nM), is very near to the plasma concentration ranges (1–18 nM) of 4-OH-Tam in breast cancer patients who are taking 20 mg of tamoxifen chronically [10, 19, 29, 40]. These results confirm that our bioassay is detecting anti-estrogenic activity at therapeutic concentrations of 4-OH-Tam.

The MCF-7 cells were also treated with endoxifen at the same concentrations. The endoxifen effectively inhibited the E2-induced PR expression in the range from 10^{-9} M to 10^{-6} M (Fig. 3a) and there was no effect of endoxifen in the absence of E2 (Fig. 3b). At a concentration of 10^{-7} M (100 nM), which is close to therapeutic concentrations (8–75 nM) in breast cancer patients on chronic tamoxifen treatment [29, 40], the endoxifen almost completely suppressed the E2-induced PR expression. The overlapping inhibition curves for 4=-OH-Tam and endoxifen indicate that the potencies for both metabolites are similar.

Endoxifen and 4-OH-Tam are both present in the human plasma of breast cancer patients taking tamoxifen. It is possible that there is a pharmacological interaction between endoxifen and 4-OH-Tam in vivo. For example, the combination may suppress the E2-induced PR expression even further than either metabolite alone. In contrast, it is also possible that a combined treatment may reduce the antagonizing effect. Therefore, we treated the MCF-7 cells with both endoxifen and 4-OH-Tam at their near-maximal effective concentrations (10^{-7} M) to

Fig. 3a, b Effects of endoxifen, pure Z-isomer, and mixed (70% Z-isomers and 30% E-isomers) form of 4-OH-Tam on the PR mRNA expression. E2 (10^{-10} M) was (**a**) present in or **(b)** absent from the culture media of MCF-7 cells. The PR mRNA expression was expressed as a percentage of the expression (E2 10^{-10} M + tamoxifen metabolite 10^{-9} M). *(Z) 4-OH-Tam* and *(M) 4-OH-Tam* stand for pure Z-isomer form and mixed form of 4-OH-Tam, respectively. The mean ± SE of three experiments is shown





determine if this altered their effectiveness in suppressing E2-induced PR expression. As shown in Fig. 4, the effect of the combination was similar to either drug alone. Although more extensive experiments will be required to fully evaluate the potential interactions between endoxifen and 4-OH-Tam, as well as other tamoxifen metabolites, the results in Fig. 4 indicate that endoxifen and 4-OH-Tam do not appear to antagonize each other—at least, at these concentrations in this biological system we used.

Measurement of tamoxifen metabolites in the culture media of the MCF-7 cells

It is possible that the reason that these two metabolites appear to be equipotent in this culture system is that the MCF-7 cells rapidly convert one metabolite to the other during the culture period. Therefore, we measured the concentrations of both endoxifen and 4-OH-Tam in the medium after 24 h of culture by HPLC (Fig. 5). In the culture media of MCF-7 cells treated with 4-OH-Tam 10^{-7} M, we detected only small amounts of endoxifen. When the cells were treated with endoxifen, 4-OH-Tam was undetectable. This suggests that the equipotency was not due to interconversion between the two drugs.

Discussion

The 4-hydroxylated metabolite of tamoxifen (4-OH-Tam) has been considered to be the most active tamoxifen metabolite, and it is not surprising that this metabolite has been frequently used to study the large range of in vitro activities of tamoxifen in laboratories around the world. Recently, we identified and characterized a new secondary metabolite of tamoxifen, endoxifen, and have shown that it has properties similar to that of 4-OH-Tam in terms of estrogen receptor binding affinity as well as anti-estrogenic action on E2-induced proliferation in MCF-7 cells [20, 40]. In a radioligand receptor competition binding assay, the relative affinity for 4-OH-Tam and endoxifen were approximately 35% and 25% of estradiol [20]. In binding assays with recombinant estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ), the relative affinities for the two receptors were essentially identical [20]. We present here that endoxifen is a potent antagonist of E2-induced expression of the progesterone receptor (PR) gene, with an inhibiting potency comparable to that of 4-OH-Tam.

The dose–response relationship in our E2-induced PR expression model in MCF-7 cells is consistent with findings from other laboratories in that the dose producing the half-maximal PR expression (ED_{50}) in this report (60 pM) was within the range of ED_{50} values (44–100 pM) reported by other authors using the same model [1]. Maximal induction was observed 24 h after drug treatment, which is consistent with a previous report [31]. These data indicate that our assay conditions are similar to those previously reported. In the presence of E2, the dose range of both 4-OH-Tam and endoxifen from 10^{-8} M (10 nM) to 10^{-7} M (100 nM) that suppressed PR expression are within, or very close to, the plasma concentration ranges found in breast cancer patients who were taking tamoxifen chronically [40], suggesting that the results obtained from our in vitro culture model are at concentrations that are therapeutically relevant in vivo.

Recently, we reported that the average human plasma concentration of endoxifen is more than six times higher than that of 4-OH-Tam in breast cancer patients taking tamoxifen (20 mg/day) [40]. Since the production of this metabolite from tamoxifen appears to be highly dependent on the genetically polymorphic enzyme, CYP2D6, the plasma concentrations of endoxifen in extensive metabolizers of CYP2D6 can be up to 14 times higher than 4-OH-Tam [11, 40]. The final unbound concentrations of these metabolites at the nuclear estrogen receptors in target tissues are difficult to estimate, although some authors reported that the tissue levels of tamoxifen and its metabolites are eight to 60 times

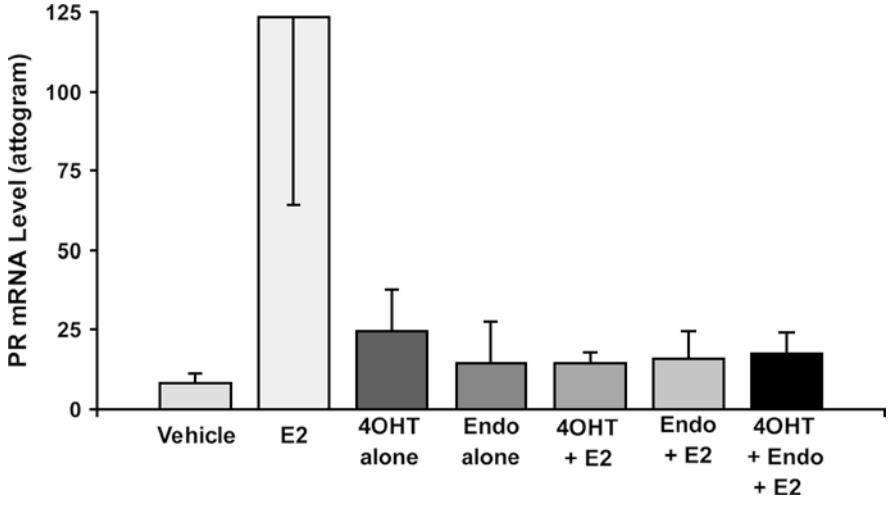


Fig. 4 Effects of the combinations of tamoxifen metabolites on the PR mRNA expression in the MCF-7 cells. Ethanol (0.1%) was used as the vehicle. The cells were treated with pure Z-isomer 4-hydroxy-tamoxifen (*4OHT*) alone (10^{-7} M), endoxifen (*Endo*) alone (10^{-7} M), or in combination with the presence or absence of E2 (10^{-10} M). The mean ± SE of three experiments is shown

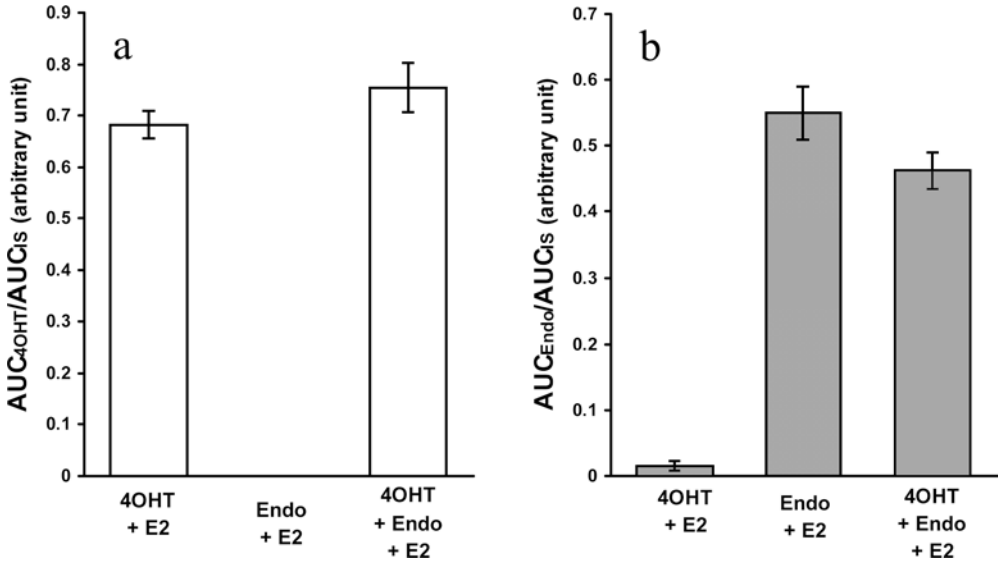


Fig. 5a, b Relative concentrations of tamoxifen metabolites in cultured media. The relative concentrations are expressed as the ratio of the area under the curve (AUC) of tamoxifen metabolites over the internal standard (IS). In the presence of E2 (10^{-10} M), the MCF-7 cells were treated for 24 h with endoxifen (*Endo*; 10^{-7} M), pure Z-isomer 4-hydroxy-tamoxifen (*4OHT*; 10^{-7} M), or a combination of both. **a** and **b** show the AUC ratio of *4OHT* or *Endo* over the internal standard, respectively. Both tamoxifen metabolites were extracted from culture media and measured using HPLC. The mean ± SE was obtained from measuring both drugs in the culture media of the two experiments

higher than in a serum [10, 30]. It is not yet known whether endoxifen concentrations influence treatment outcome and/or toxicity in patients taking tamoxifen. Nevertheless, given that 4-OH-Tam and endoxifen show comparable potency using the E2-mediated PR expression model (present data) or other models used by our group [20, 40], and that endoxifen is abundant in plasma, our data suggest that endoxifen is probably responsible for more of the in vivo tamoxifen activity than 4-OH-Tam.

In breast cancer patients, total PR levels are routinely measured to guide tamoxifen therapy and to follow breast cancer prognosis. PR exists in three isoforms, PR-A and PR-B, which are transcribed from two distinct promoters of a single gene [21], and PR-C, which appears to be the result of an alternative translation initiation start site [42]. PR-A is a truncated form of PR-B, lacking N-terminal residue that contains an activation function AF3 [38], thus, making different transcriptional activities which may result also from differential binding and recruitment of coactivators and corepressors [17]. Two PR isoforms are usually co-expressed in most PR-positive breast cancer cases. However, their ratio varies in different tissues and diseases [4, 15]. The PR-C isoform is an N-terminal truncated version that lacks the first zinc finger motif of the DNA binding domain and is transcriptionally silent by itself, but can alter the function of the other PR isoforms [41]. Further studies are needed to determine the effect of tamoxifen metabolites on the expression of specific isoforms of PR. The polymerase chain reaction (PCR) assay used here does not differentiate between the three isoforms and measures the total of all isoforms.

The Z (trans)-isomers of 4-OH-Tam is well known to be more potent anti-estrogenically than the E (cis)-isomers [23, 34, 37]. Interestingly, a mixed form of both isomers (70% Z-isomers and 30% E-isomers) 4-OH-Tam in the present study was as effective as a pure Z-isomer preparation. Although E-isomers of 4-OH-Tam is known to be estrogenic [22] or weakly anti-estrogenic [22, 33], there are several reports [22, 23, 37] that E-isomers could be converted readily to more potent anti-estrogenic Z-isomers in an in vitro cell system and Z-isomers are bound preferentially to the nuclear estrogen receptors, despite the possible conversion of Z- to E-isomer. This could be one of the possible reasons why a mixed form of both isomers 4-OH-Tam has similar anti-estrogenic actions like Z-isomers of 4-OH-Tam.

The similar pharmacological properties between 4-OH-Tam and endoxifen to date could be due to their structural similarity, especially the same position of 4-hydroxylation. Worthy of note, endoxifen is the N-demethylated product of 4-OH-Tam. Therefore, these two metabolites might differ in pharmacokinetics. For example, although NDM, an N-demethylated metabolite of tamoxifen, has very similar biological properties to those of tamoxifen, mostly because of their structural similarity, their pharmacokinetics in the subjects who took 80 mg tamoxifen once a day for 5 days was reported to be markedly different (e.g., half-life of NDM, 329 ± 53 h; tamoxifen, 118 ± 10 h) [25]. A similar difference in the pharmacokinetics of endoxifen and 4-OH-Tam has yet to be shown.

The present study showed that endoxifen antagonizes the E2-induced PR gene expression in breast cancer cells with an inhibiting potency comparable to that of 4-OH-Tam. We also have clinical evidence that endoxifen

plasma concentrations are much higher than 4-OH-Tam. These data suggest an important role of endoxifen in mediating the responses to tamoxifen, but this needs clinical testing. Since we have shown that endoxifen concentration in breast cancer patients taking tamoxifen is highly dependent on their CYP2D6 level, we will pursue additional clinical studies that will test the effects of CYP2D6 genetic variants on tamoxifen effectiveness to address the clinical role of endoxifen. Also, genome-wide expression studies are required to further characterize the pharmacology of this seemingly important metabolite.

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