0090-9556/07/3510-1942–1948$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 35, No. 10
16279/3252689
DMD 35:1942–1948, 2007
Printed in U.S.A.

Elimination of Antiestrogenic Effects of Active Tamoxifen Metabolites by Glucuronidation

Yan Zheng, Dongxiao Sun, Arun K. Sharma, Gang Chen, Shantu Amin, and Philip Lazarus

Cancer Prevention and Control (Y.Z., D.S., G.C., P.L.) and Chemical Carcinogenesis Programs (A.K.S., S.A.), Penn State Cancer Institute, Hershey, Pennsylvania; and Departments of Pharmacology (Y.Z., D.S. A.K.S., S.A., P.L.) and Public Health Sciences (G.C., P.L.), College of Medicine, Pennsylvania State University, Hershey, Pennsylvania

Received April 12, 2007; accepted July 3, 2007

ABSTRACT:

1-[4-(2-Dimethylaminoethoxy)-phenyl]-1,2-diphenylbut-1-(*Z*)-ene (tamoxifen, TAM) is a nonsteroidal antiestrogen that has been commonly used for the prevention and treatment of estrogen receptor-positive breast cancer. TAM is extensively metabolized into several primary active metabolites including 4-hydroxy-TAM (4-OH-TAM) and endoxifen. Glucuronidation is the major phase II metabolic pathway important in their excretion. Whereas high antiestrogenic activity has been reported for both 4-OH-TAM and endoxifen, studies examining the effect of glucuronide conjugation of these metabolites have not previously been performed. In the present study, the antiestrogenic activities of glucuronidated TAM metabolites were determined by examining their effect on the induction of the estrogen-responsive progesterone receptor (*PGR*) gene. 17β-Estradiol (E_2)-mediated *PGR* gene expression in MCF-7 cells was determined by real-time reverse transcriptase-polymerase chain reaction for each TAM metabolite isomer. E_2 (1×10^{-10} M) induction of PGR mRNA was 6-fold after a 12-h incubation; only unconjugated TAM metabolites inhibited this effect. A virtually identical dose-dependent inhibition of E_2-induced *PGR* gene expression was found for both the *trans*- and *cis*-isomers of 4-OH-TAM and endoxifen, with maximal inhibition attained at 1×10^{-6} M of TAM metabolite. The glucuronide conjugates of all 4-OH-TAM and endoxifen isomers exhibited no effect on E_2-mediated induction of *PGR* expression at all concentrations of TAM metabolite examined in this study. These data indicate that isomers of both 4-OH-TAM and endoxifen exhibit roughly equipotent antiestrogenic effects on E_2-induced gene expression and that glucuronide conjugates of the same metabolites effectively negate this activity. This result may have important implications in terms of both whole-body and target tissue-specific glucuronidation pathways and individual responses to TAM therapy and cancer prevention.

1-[4-(2-Dimethylaminoethoxy)-phenyl]-1,2-diphenylbut-1-(*Z*)-ene (tamoxifen, TAM) is the most commonly prescribed chemotherapeutic and chemopreventive antiestrogen for the management of estrogen receptor-positive breast cancer (Fisher et al., 1998; Osborne, 1998; Cuzick et al., 2003; Howell et al., 2003). Adjuvant TAM treatment significantly increases recurrence-free survival and overall survival in patients with estrogen receptor-positive breast cancer (Osborne, 1998; Howell et al., 2003). As a selective estrogen receptor modulator, TAM competes with estrogen for binding to the estrogen receptor and therefore inhibits tumor growth by interfering with the survival and proliferative signals regulated by estrogen. Although TAM is generally well tolerated, there is significant interindividual variability in the clinical efficacy of TAM as well as in the toxicities of TAM. For instance, TAM resistance and relapse have developed in approximately 30% of patients with estrogen receptor-positive breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1998). In addition to its antiestrogenic-related side effects such as hot flashes and vaginal bleeding (Osborne, 1998), TAM may also increase the risk for endometrial cancer (van Leeuwen et al., 1994; Rutqvist et al., 1995). TAM also has partial estrogenic effects that may be linked to reduced risk of ischemic heart disease and osteoporosis (McDonald and Stewart, 1991; Rutqvist and Mattsson, 1993). The mechanisms underlying variability in response to TAM and TAM-related toxicities are not very clear but may be related to the altered patterns of TAM metabolism.

After oral administration, TAM is extensively metabolized by phase I and phase II enzymes into several primary and secondary metabolites (Fig. 1). Two of its hydroxylated metabolites, *trans*-4-OH-TAM and 4-hydroxy-*N*-desmethyl-TAM (also known as endoxifen), exhibit high affinity for estrogen receptors, having up to 100 times more antiestrogenic activity in vitro than TAM itself or other TAM metabolites (Crewe et al., 1997; Dehal and Kupfer, 1997; Coller et al., 2002; Crewe et al., 2002; Coller, 2003; Hu et al., 2003; Desta et al., 2004). Because both 4-OH-TAM and

These studies were supported by Public Health Service (PHS) P01-68384 (National Cancer Institute) from the National Institutes of Health, Department of Health and Human Services to P.L. and by a formula grant under the Pennsylvania Department of Health's Health Research Formula Funding Program (State of PA, Act 2001-77, part of the PA Tobacco Settlement Legislation) to P.L.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.107.016279.

ABBREVIATIONS: TAM, tamoxifen, 1-[4-(2-dimethylaminoethoxy)-phenyl]-1,2-diphenylbut-1-(*Z*)-ene; 4-OH-TAM, 4-hydroxy-TAM; UGT, UDP-glucuronosyltransferase; E_2,17β-estradiol; HPLC, high-performance liquid chromatography; TEA, triethylamine; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle medium; PGR, progesterone receptor; UPLC, ultra-performance liquid chromatography; PCR, polymerase chain reaction; RT, reverse transcriptase.

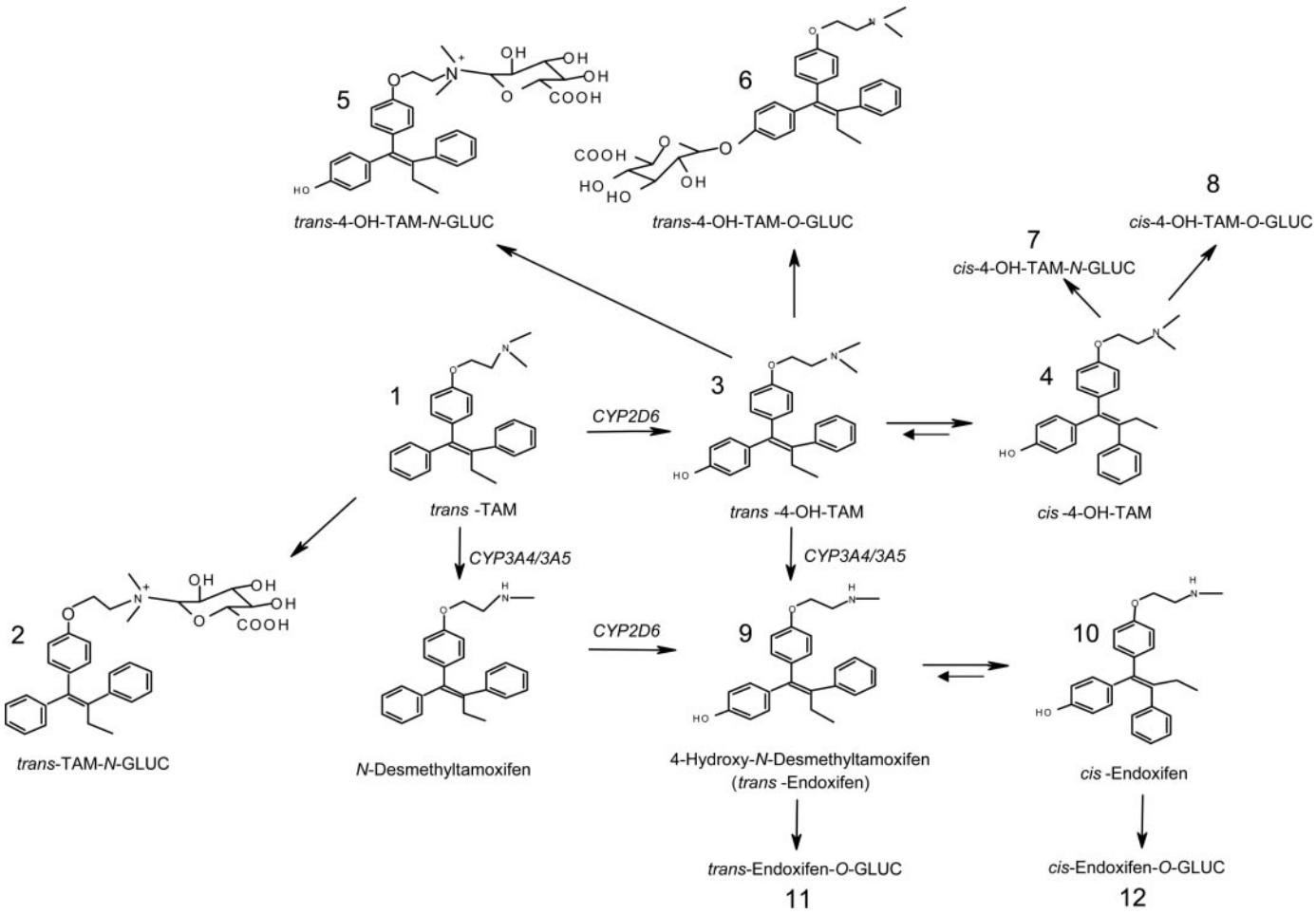

FIG. 1. Schematic diagram of TAM metabolism.

endoxifen are abundant in the serum of women treated with TAM, with endoxifen being present at 6- to 12-fold the levels of 4-OH-TAM (Stearns et al., 2003; Desta et al., 2004; Jin et al., 2005), these two TAM metabolites may be the major contributors to the antiestrogenic properties of TAM. Although *cis*-4-OH-TAM is primarily thought to be a weak estrogen agonist (Furr and Jordan, 1984), studies on individual diastereomeric forms of endoxifen have not been examined previously.

A major mode of phase II metabolism of TAM is glucuronidation catalyzed by the UDP glucuronosyltransferase (UGT) family of enzymes. TAM is excreted predominantly through the bile, a process facilitated by TAM conjugation to glucuronic acid catalyzed by UGTs (Lien et al., 1989). TAM-glucuronide conjugates have been identified in the urine, serum, and bile of TAM-treated breast cancer patients (Lien et al., 1988, 1989; Poon et al., 1993), and it has been suggested that glucuronidation within target tissues such as adipose tissue of the breast may also be important in terms of TAM metabolism and overall TAM activity (Nowell et al., 2005). Although studies have not yet been performed to examine the antiestrogenic activity of glucuronidated TAM metabolites, previous studies have demonstrated that methylation of the free phenolic group of 4-OH-TAM (producing a methyl ether) significantly decreases the affinity of 4-OH-TAM for the estrogen receptor (Allen et al., 1980).

The goal of the present study is to test the hypothesis that glucuronidation of hydroxylated TAM metabolites reduces the antiestrogenic activity of 4-OH-TAM and endoxifen. In this study, the relative antiestrogenic activities of glucuronidated conjugates of the *trans*- and *cis*-isomers of 4-OH-TAM and endoxifen compared with their unconjugated counterparts were examined. In addition, this is the first study examining the relative antiestrogenic activities of individual endoxifen isomers.

Materials and Methods

Chemicals and Materials. 17β-Estradiol (E_2), *trans*-TAM, *trans*-4-OH-TAM (98% pure), *trans*-4-OH-TAM/*cis*-4-OH-TAM mix (70%:30% ratio), UDP-glucuronic acid, and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade ammonium acetate, acetonitrile, and peptide synthesis-grade TEA were purchased from Fisher Scientific Co. (Pittsburgh, PA) and used after filtration. All media, serum, and antibiotics used for cell culture were purchased from Invitrogen (Carlsbad, CA) except for charcoal-stripped bovine calf serum (Valley Biomedical Products Inc., Winchester, VA). The RNeasy Mini Kit and QIAshredder were purchased from QIAGEN Inc. (Valencia, CA). SuperScript II RNase reverse transcriptase and oligo(dT)$_{12-18}$ primer were obtained from Invitrogen (Carlsbad, CA), and TaqMan assay reagents were purchased from Applied Biosystems (Foster City, CA). Pig liver was purchased frozen at a local grocery store and stored at −80°C until use.

Endoxifen Synthesis. Endoxifen was synthesized as described elsewhere (Sun et al., 2007). Briefly, 4-OH-TAM was refluxed with ethylchloroformate in toluene followed by treatment of the resulting intermediate (4-hydroxy-*N*-ethoxy-*N*-methyltamoxifen) with ethylene glycol, hydrazine hydrate, and potassium hydroxide at 140°C. The *trans*- and *cis*-endoxifen mixture was characterized and verified by ^{1}H nuclear magnetic resonance.

Purification and Collection of TAM Metabolite Isomers. *trans*-4-OH-TAM, *cis*-4-OH-TAM, *trans*-endoxifen, and *cis*-endoxifen were separated from the *trans*-/*cis*-4-OH-TAM (70%:30% ratio) and *trans*-/*cis*-endoxifen

stocks by HPLC as described elsewhere (Sun et al., 2007). Briefly, isocratic elution was performed using a Luna C_{18} analytical column (250 mm × 4.6 mm, 5 μ; Phenomenex, Torrance, CA) in series with a C_{18} guard column (4.0 mm × 3.0 mm, 5 μ, Phenomenex). *trans*-4-OH-TAM and *cis*-4-OH-TAM were purified by separation and elution using 20% buffer A (0.1% TEA, pH 7.4) and 80% acetonitrile, whereas *trans*-endoxifen and *cis*-endoxifen were purified with 5% buffer A (0.25% TEA, pH 7.4) and 95% acetonitrile, both at a 1 ml/min flow rate. All isomers underwent a second identical HPLC purification after initial separation to eliminate any possible contamination. Pure *trans*- and *cis*-isomers of 4-OH-TAM and endoxifen were collected individually after elution from the HPLC column and stored in DMSO at −20°C until use.

Preparation and Collection of Glucuronide Conjugates. 4-OH-TAM isomers can be glucuronidated at the *N*-amino position as well as *O*-glucuronidated at the 4-hydroxyl position, whereas endoxifen isomers are glucuronidated solely at the 4-hydroxyl position (Sun et al., 2007). The *O*-Gluc products of *trans*-4-OH-TAM, *cis*-4-OH-TAM, *trans*-endoxifen, and *cis*-endoxifen were prepared by incubation with pig liver microsomes, which were prepared essentially as described previously for human liver microsomes (Fang and Lazarus, 2004; Wiener et al., 2004b). After preincubation with alamethicin (on ice for 15 min), pig liver microsomes (100 μg) were incubated in 50 mM Tris-HCI (pH 7.4), 10 mM $MgCI_2$, 4 mM UDP-glucuronic acid with either the *trans*-4-OH-TAM/*cis*-4-OH-TAM (70:30) or *trans*-endoxifen/*cis*-endoxifen mixtures (concentration of ~100 μM each) at 37°C for 4 h in a total reaction volume of 200 μl. Reactions were terminated by addition of 200 μl of cold methanol, mixtures were centrifuged at 16,100*g* at 4°C for 10 min, and 200 μl of supernatant was injected onto the HPLC column (Gemini C_{18} 250 mm × 4.6 mm, 5 μm; Phenomenex). The gradient elution conditions for separation of *trans*- and *cis*-4-OH-TAM-*O*-Gluc were initiated with 65% buffer A and 35% acetonitrile for 15 min, followed by a subsequent linear increasing gradient to 75% acetonitrile (25% buffer A) in 1 min and then maintained at 75% acetonitrile for 10 min. The flow rate was 1 ml/min. For *trans*- and *cis*-endoxifen *O*-Gluc separation, buffer A and acetonitrile started at 67 and 33%, respectively.

For *N*-Gluc products, homogenates (50 μg) from human UGT1A4-overexpressing HK293 cells (Wiener et al., 2004a) were used in glucuronidation reactions (performed as described above in a total reaction volume of 100 μl) because pig liver cannot form *N*-glucuronidated products of TAM or its metabolites (unpublished results). For *trans*- and *cis*-4-OH-TAM-*N*-Gluc, HPLC separation started at 60% buffer A and 40% acetonitrile. As described elsewhere, no *N*-Gluc products of *trans*- or *cis*-endoxifen were detected in glucuronidation reactions using human liver microsomes or homogenates from UGT1A4-overexpressing cell lines (Sun et al., 2007). TAM-*N*-Gluc was synthesized using UGT1A4-overexpressing cell homogenates as described previously (Sun et al., 2006), and separation was started with 50% buffer A-50% acetonitrile for 16 min, with the gradient increased linearly up to 90% acetonitrile in 4 min and maintained for 10 min. Pure *trans*- and *cis*-glucuronidated products were collected after HPLC separation and purification, dissolved in DMSO, and stored at −20°C until use.

Cell Culture and Drug Treatment. MCF-7 human breast cancer cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (maintenance media) at 37°C-5% CO_2. Three days before treatment, cells were resuspended and washed twice in phosphate-buffered saline and then plated at 2×10^5 cells/dish in phenol red-free DMEM supplemented with 5% charcoal-stripped bovine calf serum (treatment media) to remove estrogens from the culture medium. For dose-response and time course studies of E_2-induced *PGR* gene expression, MCF-7 cells were treated with vehicle (0.1% DMSO) or various doses of E_2 for up to 24 h in treatment media, with cells harvested and pelleted at 0, 1, 3, 6, 12, and 24 h at 37°C-5% CO_2 after E_2 treatment and stored at −70°C until used. For studies with TAM metabolites, MCF-7 cells in treatment media were treated with E_2 (1×10^{-10} M) and TAM or TAM metabolites (1 nM–1 μM) for 12 h at 37°C and 5% CO_2. No treatment and vehicle (0.1% DMSO) controls as well as positive controls (1×10^{-10} M E_2) were included in all experiments, which were performed three times independently.

Examination of 4-OH-TAM- and Endoxifen-Glucuronides in Culture Media. A 1-ml sample of culture medium was collected before and after a 12-h incubation of MCF-7 cells with 4-OH-TAM- or endoxifen-glucuronides as described above. The 4-OH-TAM- or endoxifen-glucuronides in culture media were determined by using an Acquity UPLC system (Waters, Milford, MA). The sample preparation was as described above with 10 μl of supernatant injected onto an Acquity UPLC column (BEH C_{18} 1.7 μm, 21 mm × 100 mm; Waters). The gradient elution conditions for separation of *trans*- and *cis*-4-OH-TAM-*O*-Gluc and *trans*- and *cis*-endoxifen-*O*-Gluc was initiated with 70% buffer A (0.5 M NH_4AOc, pH = 5.0) and 30% acetonitrile for 3.5 min, followed by a subsequent linear increasing gradient to 75% acetonitrile (25% buffer A) over 0.5 min, and then maintained at 75% acetonitrile for 2 min. The flow rate was 0.3 ml/min.

***PGR* Expression Analysis.** Total RNA was extracted from treated 2×10^5 MCF-7 cells using the RNeasy Mini kit and QIAshredder (QIAGEN) as per the manufacturer's protocols and stored at −70°C until use. cDNA was synthesized in a 20-μl reaction including 100 ng of total RNA, 0.5 mmol dNTP mix, 0.5 μg oligo$(dT)_{12-18}$ primer, and 200 U of SuperScript II RNase reverse transcriptase as per the manufacturer's recommendations and stored at −70°C until use. Real-time PCR was performed using the TaqMan assay with relative quantification (ΔΔCt method) (http://www.biocompare.com/pcr/tutorial/qpcr). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as the endogenous housekeeping gene to normalize for expression for each sample. A 10-μl reaction included TaqMan Universal PCR Master Mix (1× final concentration; Applied Biosystems; Foster City, CA), primer and probe mix of either *PGR* or glyceraldehyde-3-phosphate dehydrogenase (1× final concentration; Applied Biosystems) and 40 ng of cDNA. Reactions were performed in 384-well plates using the ABI 7900 HT Sequence Detection System (Applied Biosystems). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Quantitative values of ΔΔCt and relative quantity were calculated by the feature of the ΔΔCt study option in SDS2.2.2 software (Applied Biosystems). All of the reagents and reaction conditions were preoptimized and validated by the manufacturer.

Interconversion of *trans*- and *cis*-Isomers of 4-OH-TAM or Endoxifen in Cell Culture Medium. One microliter of *trans*- or *cis* 4-OH-TAM or endoxifen (10 mM) was added to 1 ml of treatment media preadjusted to various pH values (6.0–8.5) and incubated at 37°C-5% CO_2. Aliquots of 50 μl were removed at various time points (up to 24 h) and mixed with 50 μl of acetonitrile and then centrifuged at 16,000*g* at 4°C for 10 min. Seventy microliters of supernatant was injected onto HPLC immediately after centrifugation, and relative amounts of *trans*- versus *cis*-isomers of 4-OH-TAM or endoxifen were determined. Identical experiments were also performed for glucuronidated conjugates of each of the 4-OH-TAM and endoxifen isomers. Possible pH variability of treatment media without 4-OH-TAM was measured after incubation under the same conditions. All experiments were performed three times independently.

Statistical Analysis. Student's *t* test (two-sided) was used to compare the differences of induction of PGR mRNA expression between different concentrations of test compounds. The statistical significant level was set at 0.05.

Results

Interconversions of *trans*- *and cis*-Isomers of 4-OH-TAM or Endoxifen. Previous studies have suggested that there is spontaneous interconversion between the *trans*- and *cis*-isomers of 4-OH-TAM in culture media (Katzenellenbogen et al., 1984). To examine the isomer interconversion in our experimental model, the percentage interconversion between isomers of 4-OH-TAM and endoxifen was measured in treatment media under various pH conditions for up to 24 h. As shown in Fig. 2A, approximately 2% of *trans*-4-OH-TAM was converted to *cis*-4-OH-TAM after 12 h of incubation in standard cell culture conditions at pH ≥7.5. Less than 5% interconversion was observed after a 24-h incubation at these pH values. Similarly, <2% of *cis*-4-OH-TAM was converted to *trans*-4-OH-TAM after a 24-h incubation at pH ≥7.5 (Fig. 2B). This observation is important because the biological effect of 4-OH-TAM has been suggested to be dependent upon its isomeric state: the *trans*-isomer is a potent antiestrogen whereas the *cis*-isomer has been suggested to be a weak estrogen agonist (Furr and Jordan, 1984; Stearns et al., 2003; Desta et

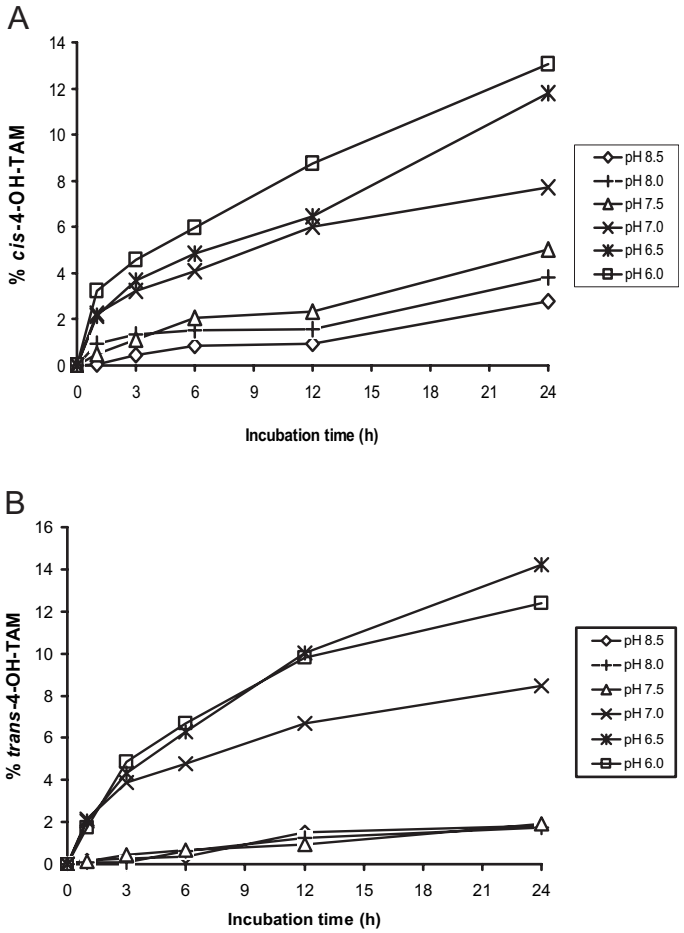

FIG. 2. Interconversion of *trans*- and *cis*-4-OH-TAM at various pH values. Shown is a representative experiment examining the interconversion between *trans*- and *cis*-isomers of 4-OH-TAM in media at various pH values. 4-OH-TAM isomers were incubated at 37°C in 5% CO_2 using treatment media of various pH values for up to 24 h. The percentage interconversion between 4-OH-TAM was determined by HPLC as indicated under *Materials and Methods*. A, conversion from *trans*- to *cis*-4-OH-TAM. B, conversion from *cis*- to *trans*-4-OH-TAM.

al., 2004; Jin et al., 2005). The percentage interconversion of the *trans*- and *cis*-isomers of endoxifen were virtually identical to that observed for 4-OH-TAM (results not shown). No interconversion was found for any of the glucuronidated *trans*- or *cis*-isomers of TAM, 4-OH-TAM, or endoxifen (results not shown). The pH values of the treatment media used in the cell culture experiments were 8.5 before incubation and 8.0 and 7.7 after 12 and 24 h of incubation, respectively. These results suggested that under the cell culture conditions used in this study, there was minimal interconversion of *trans*- and *cis*-isomers of TAM metabolites or their glucuronide conjugates.

Dose-Response and Time Course Experiment of E_2-Mediated Induction of *PGR* Gene Expression. To establish optimal conditions for estrogen induction experiments, dose-response and time course studies of E_2-induced *PGR* gene expression were conducted. MCF-7 cells were treated with 1×10^{-13} to 1×10^{-7} M E_2 for up to 24 h, and E_2-mediated induction of *PGR* gene expression was measured by real-time RT-PCR at various times after treatment. The induction of PGR mRNA was detected after a 6-h incubation using an E_2 concentration of $\geq 1 \times 10^{-11}$ M, with maximum induction (20-fold) being observed at 1×10^{-7} M E_2 after a 24-h incubation (Fig. 3). The estimated EC_{50} of E_2-induced *PGR* expression was 1.2×10^{-11} M with a 12-fold induction of PGR mRNA observed after a 12-h treatment (Fig. 3). These results are similar to that observed previously for E_2 induction of *PGR* expression in MCF-7 cells (Lim et al., 2005).

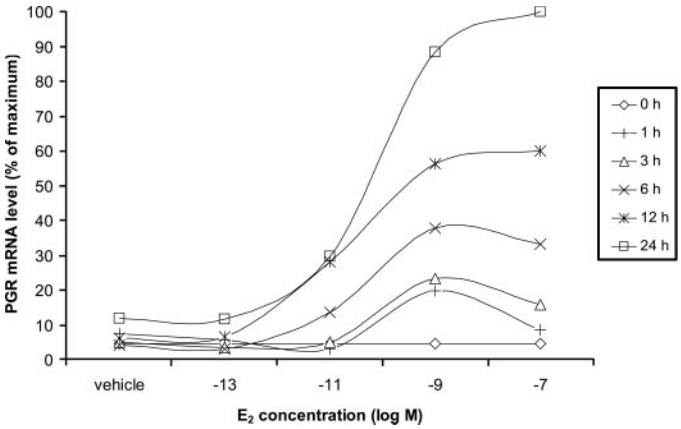

FIG. 3. Dose-response and time course of E_2-mediated induction of *PGR* gene expression in MCF-7 cells. Shown is a representative experiment examining the levels of PGR mRNA in MCF-7 cells harvested at various times after treatment with various concentrations of E_2. Total RNA was extracted at each time point for each E_2 treatment, and PGR mRNA levels were determined by RT-PCR as described under *Materials and Methods*. All values are expressed as the percentage of *PGR* expression for cells treated with 1×10^{-7} M after a 24-h incubation (i.e., the maximum inhibition of E_2-mediated induction of *PGR* expression observed in these studies). The vehicle was 0.1% DMSO.

Effects of 4-OH-TAM and Endoxifen Isomers and Their Glucuronide Conjugates on PGR mRNA Expression in the Presence of E_2. To minimize the potential confounding effects of isomeric interconversion of 4-OH-TAM and endoxifen on E_2 induction of *PGR* expression, a 12-h time point was used for induction experiments. As shown in Fig. 4, *trans*-4-OH-TAM-*O*-glucuronide (Fig. 4A) and *trans*-endoxifen-*O*-glucuronide (Fig. 4B) were stable after a 12-h incubation in media with MCF-7 cells. MCF-7 cells were incubated with 1×10^{-10} M E_2 and independently treated with each of the isomeric forms of 4-OH-TAM or endoxifen or their *O*- or *N*-glucuronide conjugates. As shown previously (Lim et al., 2005), a dose-dependent inhibition of E_2-induced *PGR* gene expression was found for both the *trans*- and *cis*-isomers of 4-OH-TAM, with maximal inhibition (complete blockage of E_2 induction of *PGR* gene expression) being attained at 1×10^{-6} M for both isomers (Fig. 5A). An identical pattern of inhibition was observed for both the *trans*- and *cis*-isomers of endoxifen (Fig. 5B). These data indicated that each of the isomers of both primary metabolites of TAM exhibited equipotent antiestrogenic effects on *PGR* gene expression in MCF-7 cells. Interestingly, similar to the effect described previously (Lim et al., 2005), TAM itself exhibited no significant antiestrogenic effect at the same dose range as that used for 4-OH-TAM or endoxifen isomers (Fig. 5A). No effect on *PGR* gene expression was observed in E_2-treated MCF-7 cells for any of the TAM, 4-OH-TAM, or endoxifen glucuronide conjugates examined in this study (Fig. 5, A and B).

In MCF-7 cells not incubated with E_2, both *trans*-4-OH-TAM and *trans*-endoxifen exhibited a slight inhibition of *PGR* gene expression in a dose-dependent manner, but this effect was not significant ($p = 0.07$; Fig. 5C). No effect on *PGR* gene expression was observed in MCF-7 cells without E_2 for *cis*-4-OH-TAM, *cis*-endoxifen, TAM, or any of the glucuronide conjugates examined.

Discussion

Whereas the major mode of metabolism of TAM is by glucuronidation through the bile (Lien et al., 1989), TAM glucuronides have been detected in the serum of TAM-treated patients (Lien et al., 1988, 1989). Although glucuronidation pathways are largely hepatic, local

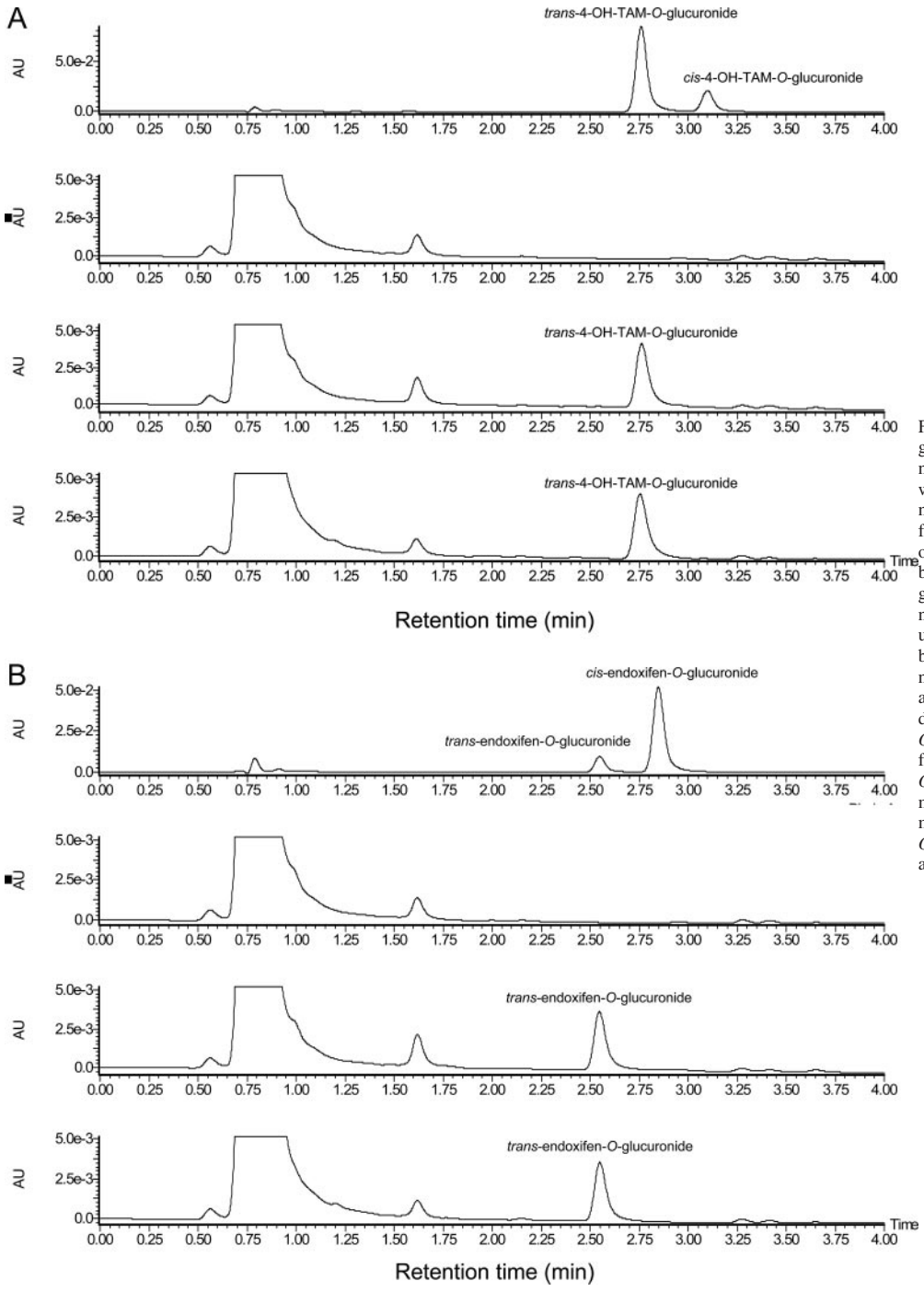

FIG. 4. The stability of *trans*-4-OH-TAM-*O*-glucuronide and *trans*-endoxifen-*O*-glucuronide in cell culture media. Cells were incubated with 5×10^{-6} M *trans*-4-OH-TAM-*O*-glucuronide (A) or *trans*-endoxifen-*O*-glucuronide (B) for 12 h at 37°C in 5% CO_2, and 1-ml amounts of media were collected before and after incubation. The stabilities of *trans*-4-OH-TAM-*O*-glucuronide and *trans*-endoxifen-*O*-glucuronide were determined by UPLC as indicated under *Materials and Methods*. A, from top to bottom: *trans*- and *cis*-4-OH-TAM-*O*-glucuronide standards; cells incubated with media alone; *trans*-4-OH-TAM-*O*-glucuronide in media before incubation; and *trans*-4-OH-TAM-*O*-glucuronide in media after incubation. B, from top to bottom: *trans*- and *cis*-endoxifen-*O*-glucuronide standards; cells incubated with media alone; *trans*-endoxifen-*O*-glucuronide in media before incubation; and *trans*-endoxifen-*O*-glucuronide in media after incubation. AU, arbitrary units.

glucuronidation activity within target tissues including the breast may impact the local pharmacological effect of TAM (Nowell et al., 2005). Therefore, it is clearly important to understand the potential impact of glucuronidation as a potential deactivation step on the pharmacological effects of TAM.

As described in previous studies, 4-OH-TAM and endoxifen exhibit up to 100 times the level of antiestrogenic activity compared with TAM itself as measured by inhibition of E_2-induced increases in cell growth, stimulation of plasminogen activator activity, or induction of *PGR* gene expression (Jordan et al., 1977; Furr and Jordan, 1984; Katzenellenbogen et al., 1984; Murphy et al., 1990; Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2005). Previous studies also showed that the antiestrogenic activities of 4-OH-TAM and endoxifen were approximately equivalent (Jordan et al., 1977; Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2005). In this report, no effect on E_2 induction of *PGR* expression was observed in cells treated with glucuronide conjugates of active TAM metabolites across the same range of TAM metabolite doses that inhibit E_2 induction in vitro and that mimic the TAM concentrations (20 mg/day) administered to women in vivo (Lee et al., 2003; Stearns et al., 2003). This pattern was observed for *trans*- and *cis*-isomers of both endoxifen- and 4-OH-TAM-glucuronides. These data strongly suggest that the glucuronide conjugates of both 4-OH-TAM and endoxifen render these metabolites inactive at relevant doses. This is particularly important with respect to residual circulating liver-initiated TAM-glu-

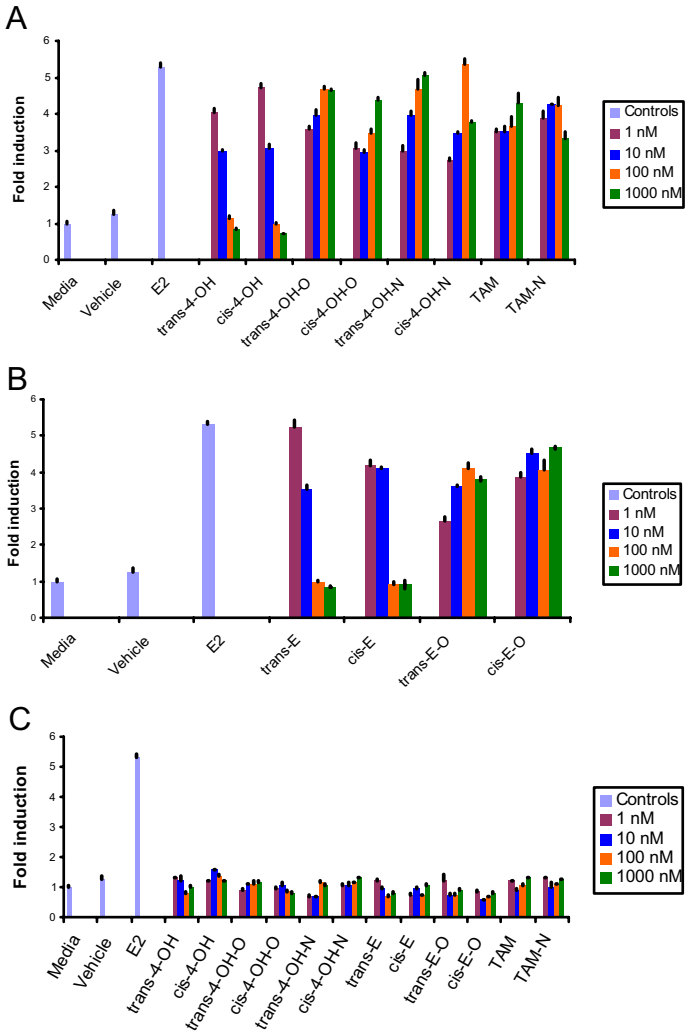

FIG. 5. Effect of TAM and TAM metabolites on *PGR* gene expression in MCF-7 cells. Cells were incubated with 1×10^{-10} M E_2 (A and B) or without E_2 (C) for 12 h at 37°C in 5% CO_2. Total RNA was extracted, and PGR mRNA levels were determined by RT-PCR as described under *Materials and Methods*. PGR mRNA levels were expressed as the fold induction of PGR mRNA levels observed for untreated cells (media). A, *PGR* gene expression in E_2-induced MCF-7 cells treated with TAM and 4-OH-TAM isomers or glucuronides; B, *PGR* gene expression in E_2-induced MCF-7 cells treated with endoxifen isomers or glucuronides; C, *PGR* gene expression in noninduced MCF-7 cells treated with TAM, 4-OH-TAM, or endoxifen isomers or their glucuronides. The E_2 positive control, the media alone, and the vehicle negative control are shown on all three panels. E_2, cells incubated with 1×10^{-10} M E_2; vehicle, cells incubated with 0.1% DMSO; *trans*-4-OH, cells incubated with *trans*-4-OH-TAM; *cis*-4-OH, cells incubated with *cis*-4-OH-TAM; *trans*-4-OH-*O*, cells incubated with *trans*-4-OH-TAM-*O*-glucuronide; *cis*-4-OH-*O*, cells incubated with *cis*-4-OH-TAM-*O*-glucuronide; *trans*-4-OH-*N*, cells incubated with *trans*-4-OH-TAM-*N*-glucuronide; *cis*-4-OH-*N*, cells incubated with *cis*-4-OH-TAM-*N*-glucuronide; *trans*-E, cells incubated with *trans*-endoxifen; *cis*-E, cells incubated with *cis*-endoxifen; *trans*-E-*O*, cells incubated with *trans*-endoxifen-*O*-glucuronide; *cis*-E-*O*, cells incubated with *cis*-endoxifen-*O*-glucuronide; TAM, cells incubated with TAM; TAM-*N*, cells incubated with TAM-*N*-glucuronide. The figure legend indicates the concentration of TAM or TAM metabolite used in this experiment. The mean ± S.E. is shown for three experiments.

curonides that were not excreted into bile as well as adipose-dependent glucuronidation effects, because alterations in glucuronidation capacity could therefore significantly impact the local pharmacology within target tissues.

Previous studies examining the antiestrogenic properties of TAM metabolite isomers have been hindered by the interconversion between the *cis*- and *trans*-isomers of TAM and TAM metabolites shown to occur in previous experimental systems (Jordan et al., 1981; Lieberman et al., 1983; Katzenellenbogen et al., 1984). The present study is the first to examine the antiestrogenic properties of relatively pure isomers of both 4-OH-TAM and endoxifen. This was made possible by the fact that results from the present study demonstrated that the interconversion properties of TAM metabolite isomers are highly pH-dependent, with decreasing interconversion occurring at higher pH values (7.8–8.4). Although significant interconversion (up to 14%) was observed between *trans*- and *cis*-isomers at lower pH values, there was <3% conversion between *trans*- and *cis*-isomers for both 4-OH-TAM and endoxifen after a 12-h incubation in the cell culture media used in this study. This level of interconversion remained relatively constant over the time course examined with or without the presence of E_2 and/or TAM metabolites. This contrasts with the up to 18% isomer interconversion observed over a similar incubation time in previous study (Katzenellenbogen et al., 1984). Therefore, unlike previous in vitro cell culture systems where the pH may not have been optimal to prevent interconversion between isomers, the antiestrogenic properties of relatively pure isomers of TAM metabolites were examined in this study.

Previous studies have shown that the *cis*- and *trans*-isomers of 4-OH-TAM exhibit similar patterns of antiestrogenic activities. Interpretation of results from previous studies was difficult because of the possible interconversion between isomers. It was originally suggested that because *cis*-TAM is estrogenic, the antiestrogenic properties of *cis*-4-OH-TAM in a rat uterine weight test model may be due to interconversion to its *trans*-form (Jordan et al., 1981). A later study using a prolactin synthesis model demonstrated a stronger antiestrogenic effect for *trans*-4-OH-TAM compared with *cis*-4-OH-TAM and that neither isomer exhibited estrogenic properties, but these data were also confounded by the possibility of interconversion between isomers (Lieberman et al., 1983). Similarly, suppression of MCF-7 cell growth and E_2-induced stimulation of plasminogen activator activity by the *cis*-isomer of 4-OH-TAM was suggested to be due to isomer interconversion (Katzenellenbogen et al., 1984). This suggestion was confirmed in studies of fixed-ring *trans*- and *cis*-isomers of TAM and 4-OH-TAM, which prevented isomer interconversion (Murphy et al., 1990). The inhibition of E_2-mediated induction of *PGR* gene expression was roughly equivalent for the *trans*- versus *cis*-isomers of 4-OH-TAM in previous studies (Lim et al., 2005), a pattern similar to that observed in the present study for both 4-OH-TAM and endoxifen. These data suggest that the *trans*- and *cis*-isomers of these active TAM metabolites exhibit similar properties in terms of inhibition of E_2-mediated induction of *PGR* gene expression and other E_2-mediated cellular properties.

This is the first study to examine the antiestrogenic effects of individual isomers of endoxifen. Results from the present study demonstrate that both the *trans*- and *cis*-isomers of endoxifen exhibit antiestrogenic activity. Similar levels of reduction in E_2-induced *PGR* gene expression were observed for the two endoxifen isomers at all TAM metabolite doses examined in this study. Interestingly, the same level of inhibition of E_2-induced expression of PGR was observed for endoxifen isomers as for isomers of 4-OH-TAM. This is similar to the antiestrogenic properties observed for the two TAM metabolites in previous studies (Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2005). Also similar to that observed in previous studies (Katzenellenbogen et al., 1984) is the fact that TAM exhibited no effect on E_2-induced *PGR* expression in MCF-7 cells at all TAM concentrations tested. This result further supports the fact that the TAM metabolites, 4-OH-TAM and endoxifen, and not TAM itself, are the active antiestrogens in women treated with TAM.

Consistent with the antiestrogenic properties of the *trans*-isomer of 4-OH-TAM and endoxifen, *PGR* gene expression was slightly inhib-

ited when MCF-7 cells were incubated in the absence of E_2. In contrast, there was no effect on *PGR* gene expression by the corresponding *cis*-isomers. This is consistent with a decreased antiestrogenic potential for the *cis*-isomers of these TAM metabolites.

The results from this study suggest that glucuronidation is an important detoxifying and deactivating metabolic pathway for TAM. Because TAM is an important antiestrogen for the treatment and prevention of estrogen-dependent breast cancer, individuals with higher glucuronidation capacity may therefore deactivate and detoxify TAM at higher rates, potentially increasing risk of breast cancer occurrence and/or recurrence due to lowered drug efficacy. It is also possible that such variations in glucuronidation capacity may alter the risk for TAM-related toxicities. Studies examining the role of UGT pharmacogenetics in different individuals could provide us with important insight into the role of glucuronidation in patient response to TAM.

Acknowledgments. We thank the Organic Synthesis Core Facility at the Penn Sate College of Medicine for endoxifen synthesis and the Penn Sate College of Medicine Functional Genomics Core Facility for access to equipment for real-time PCR analysis.

References

Allen KE, Clark ER, and Jordan VC (1980) Evidence for the metabolic activation of non-steroidal antioestrogens: a study of structure-activity relationships. *Br J Pharmacol* **71:**83–91.

Coller JK (2003) Oxidative metabolism of tamoxifen to *Z*-4-hydroxy-tamoxifen by cytochrome P450 isoforms: an appraisal of in vitro studies. *Clin Exp Pharmacol Physiol* **30:**845–848.

Coller JK, Krebsfaenger N, Klein K, Endrizzi K, Wolbold R, Lang T, Nussler A, Neuhaus P, Zanger UM, Eichelbaum M, et al. (2002) The influence of CYP2B6, CYP2C9 and CYP2D6 genotypes on the formation of the potent antioestrogen *Z*-4-hydroxy-tamoxifen in human liver. *Br J Clin Pharmacol* **54:**157–167.

Crewe HK, Ellis SW, Lennard MS, and Tucker GT (1997) Variable contribution of cytochromes P450 2D6, 2C9 and 3A4 to the 4-hydroxylation of tamoxifen by human liver microsomes. *Biochem Pharmacol* **53:**171–178.

Crewe HK, Notley LM, Wunsch RM, Lennard MS, and Gillam EM (2002) Metabolism of tamoxifen by recombinant human cytochrome P450 enzymes: formation of the 4-hydroxy, 4′-hydroxy and *N*-desmethyl metabolites and isomerization of *trans*-4-hydroxytamoxifen. *Drug Metab Dispos* **30:**869–874.

Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, and Boyle P (2003) Overview of the main outcomes in breast-cancer prevention trials. *Lancet* **361:**296–300.

Dehal SS and Kupfer D (1997) CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. *Cancer Res* **57:**3402–3406.

Desta Z, Ward BA, Soukhova NV, and Flockhart DA (2004) Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. *J Pharmacol Exp Ther* **310:**1062–1075.

Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* **351:**1451–1467.

Fang JL and Lazarus P (2004) Correlation between the UDP-glucuronosyltransferase (UGT1A1) TATAA box polymorphism and carcinogen detoxification phenotype: significantly decreased glucuronidating activity against benzo(*a*)pyrene-7,8-dihydrodiol(−) in liver microsomes from subjects with the UGT1A1*28 variant. *Cancer Epidemiol Biomarkers Prev* **13:**102–109.

Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, et al. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* **90:**1371–1388.

Furr BJ and Jordan VC (1984) The pharmacology and clinical uses of tamoxifen. *Pharmacol Ther* **25:**127–205.

Howell A, Howell SJ, and Evans DG (2003) New approaches to the endocrine prevention and treatment of breast cancer. *Cancer Chemother Pharmacol* **52 (Suppl 1:)**S39–S44.

Hu Y, Dehal SS, Hynd G, Jones GB, and Kupfer D (2003) CYP2D6-mediated catalysis of tamoxifen aromatic hydroxylation with an NIH shift: similar hydroxylation mechanism in chicken, rat and human liver microsomes. *Xenobiotica* **33:**141–151.

Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee KH, Skaar T, Storniolo AM, Li L, Araba A, et al. (2005) CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J Natl Cancer Inst* **97:**30–39.

Johnson MD, Zuo H, Lee KH, Trebley JP, Rae JM, Weatherman RV, Desta Z, Flockhart DA, and Skaar TC (2004) Pharmacological characterization of 4-hydroxy-N-desmethyl tamoxifen, a novel active metabolite of tamoxifen. *Breast Cancer Res Treat* **85:**151–159.

Jordan VC, Collins MM, Rowsby L, and Prestwich G (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J Endocrinol* **75:**305–316.

Jordan VC, Haldemann B, and Allen KE (1981) Geometric isomers of substituted triphenylethylenes and antiestrogen action. *Endocrinology* **108:**1353–1361.

Katzenellenbogen BS, Norman MJ, Eckert RL, Peltz SW, and Mangel WF (1984) Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxy-tamoxifen isomers in MCF-7 human breast cancer cells. *Cancer Res* **44:**112–119.

Lee KH, Ward BA, Desta Z, Flockhart DA, and Jones DR (2003) Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: application to a clinical trial. *J Chromatogr B Analyt Technol Biomed Life Sci* **791:**245–253.

Lieberman ME, Gorski J, and Jordan VC (1983) An estrogen receptor model to describe the regulation of prolactin synthesis by antiestrogens in vitro. *J Biol Chem* **258:**4741–4745.

Lien EA, Solheim E, Kvinnsland S, and Ueland PM (1988) Identification of 4-hydroxy-*N*-desmethyltamoxifen as a metabolite of tamoxifen in human bile. *Cancer Res* **48:**2304–2308.

Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S, and Ueland PM (1989) Distribution of 4-hydroxy-*N*-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. *Cancer Res* **49:**2175–2183.

Lim YC, Desta Z, Flockhart DA, and Skaar TC (2005) Endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. *Cancer Chemother Pharmacol* **55:**471–478.

McDonald CC and Stewart HJ (1991) Fatal myocardial infarction in the Scottish adjuvant tamoxifen trial; The Scottish Breast Cancer Committee. *BMJ* **303:**435–437.

Murphy CS, Langan-Fahey SM, McCague R, and Jordan VC (1990) Structure-function relationships of hydroxylated metabolites of tamoxifen that control the proliferation of estrogen-responsive T47D breast cancer cells in vitro. *Mol Pharmacol* **38:**737–743.

Nowell SA, Ahn J, Rae JM, Scheys JO, Trovato A, Sweeney C, MacLeod SL, Kadlubar FF, and Ambrosone CB (2005) Association of genetic variation in tamoxifen-metabolizing enzymes with overall survival and recurrence of disease in breast cancer patients. *Breast Cancer Res Treat* **91:**249–258.

Osborne CK (1998) Tamoxifen in the treatment of breast cancer. *N Engl J Med* **339:**1609–1618.

Poon GK, Chui YC, McCague R, Llnning PE, Feng R, Rowlands MG, and Jarman M (1993) Analysis of phase I and phase II metabolites of tamoxifen in breast cancer patients. *Drug Metab Dispos* **21:**1119–1124.

Rutqvist LE, Johansson H, Signomklao T, Johansson U, Fornander T, and Wilking N (1995) Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies: Stockholm Breast Cancer Study Group. *J Natl Cancer Inst* **87:**645–651.

Rutqvist LE and Mattsson A (1993) Cardiac and thromboembolic morbidity among postmenopausal women with early-stage breast cancer in a randomized trial of adjuvant tamoxifen: the Stockholm Breast Cancer Study Group. *J Natl Cancer Inst* **85:**1398–1406.

Stearns V, Johnson MD, Rae JM, Morocho A, Novielli A, Bhargava P, Hayes DF, Desta Z, and Flockhart DA (2003) Active tamoxifen metabolite plasma concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. *J Natl Cancer Inst* **95:**1758–1764.

Sun D, Chen G, Dellinger RW, Duncan K, Fang JL, and Lazarus P (2006) Characterization of tamoxifen and 4-hydroxytamoxifen glucuronidation by human UGT1A4 variants. *Breast Cancer Res* **8:**R50.

Sun D, Sharma AK, Dellinger RW, Blevins-Primeau AS, Balliet R, Chen G, Boyiri T, Amin S, and Lazarus P (2007) Glucuronidation of active tamoxifen metabolites by the human UDP-glucuronosyltransferases (UGTs). *Drug Metab Dispos*, in press.

van Leeuwen FE, Benraadt J, Coebergh JW, Kiemeney LA, Gimbrere CH, Otter R, Schouten LJ, Damhuis RA, Bontenbal M, Diepenhorst FW, et al. (1994) Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet* **343:**448–452.

Wiener D, Doerge DR, Fang JL, Upadhyaya P, and Lazarus P (2004a) Characterization of *N*-glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human liver: importance of UDP-glucuronosyltransferase 1A4. *Drug Metab Dispos* **32:**72–79.

Wiener D, Fang JL, Dossett N, and Lazarus P (2004b) Correlation between UDP-glucuronosyltransferase genotypes and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone glucuronidation phenotype in human liver microsomes. *Cancer Res* **64:**1190–1196.

Address correspondence to: Dr. Philip Lazarus, Division of Population Sciences and Cancer Prevention, Penn State Cancer Institute, Department of Pharmacology, MC-H069, Penn State College of Medicine, 500 University Dr., Hershey, PA 17033. E-mail: plazarus@psu.edu