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ELIMINATION OF ANTI-ESTROGENIC EFFECTS OF ACTIVE TAMOXIFEN METABOLITES BY GLUCURONIDATION

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Abbreviation: DMEM, Dulbecco's modified Eagle medium; DMSO, Dimethyl sulfoxide, E₂, 17 β -estradiol; ER, estrogen receptor; HPLC, high performance liquid chromatography; PGR, progesterone receptor; RT-PCR, real-time polymerase chain reaction; TAM, tamoxifen; 4-OH-TAM, 4-hydroxy-TAM; TEA, triethylamine; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC, ultra performance liquid chromatography.

ABSTRACT

TAM is a nonsteroidal anti-estrogen 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-OH-TAM and endoxifen. Glucuronidation is the major phase II metabolic pathway important in their excretion. While high anti-estrogenic 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 anti-estrogenic activities of glucuronidated TAM metabolites were determined by examining their effect on the induction of the estrogen-responsive PGR gene. E₂-mediated PGR gene expression in MCF-7 cells was determined by real-time RT-PCR for each TAM metabolite isomer. E₂ (1x10⁻¹⁰ M) induction of PGR mRNA was 6-fold after 12 h incubation; only unconjugated TAM metabolites inhibited this effect. A virtually identical dose-dependent inhibition of E₂-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 x10⁻⁶ M of TAM metabolite. The glucuronide conjugates of all 4-OH-TAM and endoxifen isomers exhibited no effect on E₂-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 anti-estrogenic effects on E₂-induced gene expression and that glucuronide conjugates of the same metabolites effectively negate this activity. This may have important implications in terms of both whole-body and target-tissue-specific glucuronidation pathways and individual response to TAM therapy and cancer prevention.

Introduction

TAM, (1-[4-(2-dimethylaminoethoxy)-phenyl]-1,2-diphenylbut-1-(Z)-ene) is the most commonly prescribed chemotherapeutic and chemopreventive anti-estrogen 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 estrogen receptor-positive breast cancer patients (Osborne, 1998; Howell et al., 2003). As a selective estrogen receptor modulator, TAM competes with estrogen for binding to the ER 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 inter-individual variability in the clinical efficacy of TAM as well as in the toxicities of TAM. For instance, TAM resistance and relapse have been developed in about 30% of the estrogen receptor-positive breast cancer patients (1998). In addition to its anti-estrogenic-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.

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After oral administration, TAM is extensively metabolized by phase I and phase II enzymes into several primary and secondary metabolites (Figure 1). Two of its hydroxylated metabolites, *trans*-4-OH-TAM and 4-hydroxy-*N*-desmethyl-TAM (also known as endoxifen) exhibit high affinity for ER, exhibiting up to 100 times more anti-estrogenic activity *in vitro* than TAM itself as well as other TAM metabolites (Crewe et al., 1997; Dehal and Kupfer, 1997; Collier et al., 2002; Crewe et al., 2002; Collier, 2003; Hu et al., 2003; Desta et al., 2004). Since both 4-OH-TAM and endoxifen are abundant in the serum of women treated with TAM, with endoxifen present at 6-12-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 TAM's anti-estrogenic properties. While *cis*-4-OH-TAM is primarily thought to be an estrogen agonist with a weak estrogenic effect (Furr and Jordan, 1984), studies on individual diastereomeric forms of endoxifen have not been previously examined.

A major mode of phase II metabolism of TAM is by glucuronidation catalyzed by the 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; Lien et al., 1989; Poon et al., 1993), and it has been suggested that glucuronidation within target tissues like 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 examining the anti-estrogenic activity of glucuronidated TAM metabolites, previous studies have

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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 anti-estrogenic activity of 4-OH-TAM and endoxifen. In this study, the relative anti-estrogenic activities of glucuronidated conjugates of the *trans* and *cis* isomers of 4-OH-TAM and endoxifen as compared to their unconjugated counterparts were examined. In addition, this is the first study examining the relative anti-estrogenic activities of individual endoxifen isomers.

Materials and Methods

Chemicals and materials. 17 β -estradiol (E₂), *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, USA). HPLC-grade ammonium acetate, acetonitrile, and peptide synthesis-grade TEA were purchased from Fisher Scientific (Pittsburgh, PA) and used after filtration. All media, serum and antibiotics used for cell culture were purchased from Gibco (Grand Island, NY) except for charcoal-stripped bovine calf serum (Valley Biomedical Products Inc., Winchester, VA). RNeasy[®] Mini Kit and QIAshredder[™] were purchased from Qiagen Inc. (Valencia, CA, USA). SuperScript[™] II RNase reverse transcriptase and Oligo (dT)₁₂₋₁₈ primer were obtained from Invitrogen (Carlsbad, CA) while TaqMan[®] assay reagents were purchased from Applied Biosystems (Foster City, CA). Pig liver was purchased frozen at a local grocery and stored at -80°C until use.

Endoxifen synthesis. Endoxifen was synthesized as previously described (Sun et al., Submitted). Briefly, 4-OH-TAM was refluxed with ethylchloroformate in toluene followed by treatment of the resulting intermediate (4-hydroxy-*N*-ethoxy-*N*-methyltamoxifen) with ethyleneglycol, hydrazine hydrate, and potassium hydroxide at 140°C. The *trans*- and *cis*-endoxifen mixture was characterized and verified by ¹H NMR.

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-

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OH-TAM (70%:30% ratio) and *trans*:-*cis*-endoxifen stocks by HPLC as previously described (Sun et al., Submitted). Briefly, isocratic elution was performed using a Luna C₁₈ analytical column (250 mm × 4.6 mm, 5 μ; Phenomenex, Torrance, CA) in series with a C₁₈ guard column (4.0 mm × 3.0 mm, 5 μ, Phenomenex, Torrance, CA). *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, while *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, while endoxifen isomers are glucuronidated solely at the 4-hydroxyl-position (Sun et al., Submitted). The *O*-Gluc products of *trans*-4-OH-TAM, *cis*-4-OH-TAM, *trans*-endoxifen and *cis*-endoxifen were prepared by incubating with pig liver microsomes, which were prepared essentially as previously described for human liver microsomes (Fang and Lazarus, 2004; Wiener et al., 2004b). After pre-incubation with alamethicin (on ice for 15 min), pig liver microsomes (100 μg) were incubated in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4 mM UDPGA with either the *trans*-4-OH-TAM:*cis*-4-OH-TAM (70:30) or *trans*-endoxifen:*cis*-endoxifen mixtures (~100 μM each) at 37°C for 4 h in a total reaction volume of 200 μL. Reactions were terminated by addition of 200

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μ L cold methanol, mixtures were centrifuged at 16,100 g at 4°C for 10 min, and 200 μ L of supernatant were injected onto HPLC column (Gemini, C₁₈ 250 mm \times 4.6 mm, 5 μ m, Phenomenex, Torrance, CA). The gradient elution conditions for separation of *trans*- and *cis*-4-OH-TAM-O-Gluc was 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 μ g) from human UGT1A4-over-expressing HK293 cells (Wiener et al., 2004a) were used in glucuronidation reactions (performed as described above in a total reaction volume of 100 μ L) since 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 previously, no *N*-Gluc products of *trans*- or *cis*-endoxifen were detected in glucuronidation reaction using human liver microsomes or homogenates from UGT1A4-over-expressing cell lines (Sun et al., Submitted). TAM-*N*-Gluc was synthesized using UGT1A4-over-expressing cell homogenates as previously described (Sun et al., 2006) and separation 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.

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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% CO₂. Three days prior to 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 E₂-induced PGR gene expression, MCF-7 cells were treated with vehicle (0.1% DMSO) or various doses of E₂ 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% CO₂ after E₂ treatment, and stored at -70°C until used. For studies with TAM metabolites, MCF-7 cells in treatment media were treated with E₂ (1×10^{-10} M) and TAM or TAM metabolites (1 nM – 1 μ M) for 12 h at 37°C and 5% CO₂. No-treatment and vehicle (0.1% DMSO) controls as well as positive controls (1×10^{-10} M E₂) were included in all experiments, with experiments performed three times in independent experiments.

Examination of 4-OH-TAM and endoxifen-glucuronides in culture media. A 1 mL sample of culture media 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 μ L of supernatant injected onto an Acquity UPLC® column (BEH, C₁₈ 1.7 μ m, 21mm \times 100 mm, Waters, Milford, MA). The gradient elution conditions for separation of

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trans- and *cis*-4-OH-TAM-O-Gluc and *trans*- and *cis*-endoxifen-O-Gluc was initiated with 70% buffer A (0.5 M NH₄AOc, 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 2×10⁵ MCF-7 cells using the RNeasy[®] Mini kit and QIAshredder[™] (Qiagen Inc.) as per the manufacturer's protocols and stored at -70°C until use. cDNA was synthesized in a 20 µL reaction including 100 ng total RNA, 0.5 mmol dNTP mix, 0.5 µg Oligo (dT)₁₂₋₁₈ primer, and 200 U SuperScript[™] II RNase reverse transcriptase as per the manufacturer recommendations and stored at -70°C until use. Real-time PCR was performed using the TaqMan assay with relative quantification (Delta-Delta Ct method) (<http://www.biocompare.com/pcr/tutorial/qpcr/statis/delta.asp>). The 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 (1x final concentration; Applied Biosystems; Foster City, CA), primer and probe mix of either PGR or GAPDH (1X 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 sec, 60°C for 1 min. Quantitative values of $\Delta\Delta C_t$ and RQ were calculated by the feature of the $\Delta\Delta C_t$ study option in SDS2.2.2 software (Applied Biosystems). All the reagents and reaction conditions were pre-optimized and validated by the manufacturer.

Interconversion of *trans*- and *cis*-isomers of 4-OH-TAM or endoxifen in cell culture medium. One μL of *trans*- or *cis* 4-OH-TAM or endoxifen (10 mM) was added to 1 mL treatment media pre-adjusted to various pHs (6.0 – 8.5) and incubated at 37°C, 5% CO₂. Fifty μL aliquots were removed at various time points (up to 24 h) and mixed with 50 μL acetonitrile, and then centrifuged at 16,000 g at 4°C for 10 min. Seventy μL 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 incubating under the same conditions. All experiments were performed 3 times in independent experiments.

Statistical analysis. The 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 percent 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 Figure 2 (panel A), 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 24 h incubation at these pHs. Similarly, < 2% of *cis*-4-OH-TAM was converted to *trans*-4-OH-TAM after 24 h incubation at pH ≥ 7.5 (Figure 2, panel B). This observation is important since the biological effect of 4-OH-TAM has been suggested to be dependent upon its isomeric state - the *trans*-isomer is a potent anti-estrogen whereas the *cis*-isomer has been suggested to be a weak estrogen agonist (Furr and Jordan, 1984; Stearns et al., 2003; Desta et al., 2004; Jin et al., 2005). The percent 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 of the treatment media used in the cell culture experiments was 8.5 before incubation, and was pH 8.0 and 7.7 after 12 and 24 h 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 E₂-mediated induction of PGR gene expression. In order to establish optimal conditions for estrogen induction experiments, dose-response and time-course studies of E₂-induced PGR gene expression were conducted. MCF-7 cells were treated with 1×10^{-13} – 1×10^{-7} M E₂ for up to 24 h, and E₂-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 E₂ concentration of $\geq 1 \times 10^{-11}$ M, with maximum induction (20-fold) observed at 1×10^{-7} M E₂ after a 24 h incubation (Figure 3). The estimated EC₅₀ of E₂-induced PGR expression was 1.2×10^{-11} M with a 12-fold induction of PGR mRNA observed after 12 h treatment (Figure 3). These results are similar to that observed previously for E₂ induction of PGR expression in MCF-7 cells (Lim et al., 2005).

Effects of 4-OH-TAM and endoxifen isomers and their glucuronide conjugates on PGR mRNA expression in the presence of E₂. To minimize the potential confounding effects of isomeric interconversion of 4-OH-TAM and endoxifen on E₂ induction of PGR expression, a 12 h time-point was used for induction experiments. As shown in Figure 4, *trans*-4-OH-TAM-O-glucuronide (panel A) and *trans*-endoxifen-O-glucuronide (panel B) were stable after a 12 h incubation in media with MCF-7 cells. MCF-7 cells were incubated with 1×10^{-10} M E₂ and independently treated with each of the isomeric forms of 4-OH-TAM or endoxifen or their *O*- or *N*-

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glucuronide conjugates. As shown previously (Lim et al., 2005), a dose-dependent inhibition of E₂-induced PGR gene expression was found for both the *trans* and *cis* isomers of 4-OH-TAM, with maximal inhibition (complete blockage of E₂ induction of PGR gene expression) attained at 1x10⁻⁶ M for both isomers (Figure 5, panel A). An identical pattern of inhibition was observed for both the *trans* and *cis* isomers of endoxifen (Figure 5, panel B). These data indicated that each of the isomers of both primary metabolites of TAM exhibited equipotent anti-estrogenic effects on PGR gene expression in MCF-7 cells. Interestingly, similar to that described previously (Lim et al., 2005), TAM itself exhibited no significant anti-estrogenic effect at the same dose range as that used for 4-OH-TAM or endoxifen isomers (Figure 5, panel A). No effect on PGR gene expression was observed in E₂-treated MCF-7 cells for any of the TAM, 4-OH-TAM or endoxifen glucuronide conjugates examined in this study (Figure 5, panels A and B).

In MCF-7 cells not incubated with E₂, 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 ($p = 0.07$; Figure 5, panel C). No effect on PGR gene expression was observed in MCF-7 cells without E₂ for *cis*-4-OH-TAM, *cis*-endoxifen, TAM, or any of the glucuronide conjugates examined.

Discussion

While 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; Lien et al., 1989). While glucuronidation pathways are largely hepatic, local 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 anti-estrogenic activity as compared to TAM itself as measured by inhibition of E₂-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., 2004; Lim et al., 2005). Previous studies also showed that the anti-estrogenic 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., 2004; Lim et al., 2005). In this report, no effect on E₂ 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 E₂ 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-

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

Previous studies examining the anti-estrogenic properties of TAM metabolite isomers have been hindered by the fact that 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 anti-estrogenic 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 pHs (7.8-8.4). While significant interconversion (up to 14%) was observed between *trans* and *cis* isomers at lower pH's, there was < 3% conversion between *trans* and *cis* isomers for both 4-OH-TAM and endoxifen after 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 E₂ and/or TAM metabolite. 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

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prevent interconversion between isomers, the anti-estrogenic 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 anti-estrogenic activities. Interpretation of results from previous studies was made difficult due to possible interconversion between isomers. It was originally suggested that since *cis*-TAM is estrogenic, the anti-estrogenic 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). Later study using a prolactin synthesis model demonstrated a stronger anti-estrogenic effect for *trans*-4-OH-TAM as compared to *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 E₂-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 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 E₂-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 as well as endoxifen. These data suggest that the *trans* and *cis* isomers of these active TAM metabolites exhibit similar properties in terms of inhibition of E₂-mediated induction of PGR gene expression and other E₂-mediated cellular properties.

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This is the first study to examine the anti-estrogenic effects of individual isomers of endoxifen. Results from the present study demonstrate that both the *trans* and *cis* isomers of endoxifen exhibit anti-estrogenic activity. Similar levels of reduction in E₂-induced PGR gene expression was observed for the two endoxifen isomers at all TAM metabolite doses examined in this study. Interestingly, the same level of inhibition of E₂-induced expression of PGR was observed for endoxifen isomers as for isomers of 4-OH-TAM. This is similar to the anti-estrogenic 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 E₂-induced PGR expression in MCF-7 cells at all TAM concentrations tested. This further supports the fact that it is the TAM metabolites, 4-OH-TAM and endoxifen, and not TAM itself, which are the active anti-estrogens in women treated with TAM.

Consistent with the anti-estrogenic properties of the *trans* isomer of 4-OH-TAM and endoxifen, PGR gene expression was slightly inhibited when MCF-7 cells were incubated in the absence of E₂. In contrast, there was no effect on the PGR gene expression by the corresponding *cis* isomers. This is consistent with a decreased anti-estrogenic 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. Since TAM is an important

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anti-estrogen 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 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.

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Footnotes

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Legends for Figures

Figure 1. Schematic of TAM metabolism.

Figure 2. Interconversion of *trans*- and *cis*-4-OH-TAM at varying pH. Shown is a representative experiment examining the interconversion between *trans* and *cis* isomers of 4-OH-TAM in media at varying pHs. 4-OH-TAM isomers were incubated at 37°C in 5% CO₂ using treatment media of varying pH for up to 24 h. The percent interconversion between 4-OH-TAM was determined by HPLC as indicated in the Methods. **A.** Conversion from *trans*- to *cis*-4-OH-TAM. **B.** Conversion from *cis*- to *trans*-4-OH-TAM.

Figure 3. Dose-response and time-course of E₂-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 E₂. Total RNA was extracted at each time point for each E₂ treatment and PGR mRNA levels were determined by RT-PCR as described in the Methods. All values are expressed as the percentage of PGR expression for cells treated with 1x10⁻⁷ M after 24 h incubation (i.e., the maximum inhibition of E₂-mediated induction of PGR expression observed in these studies). The vehicle was 0.1% DMSO.

Figure 4. The stability of *trans*-4-OH-TAM-O-glucuronide and *trans*-endoxifen-O-glucuronide in cell culture media. Cells were incubated with 5x10⁻⁶ M *trans*-4-OH-TAM-O-glucuronide (A) or *trans*-endoxifen-O-glucuronide (B) for 12 h at 37°C in 5%

CO₂. One mL media were collected before and after incubation. The stability of *trans*-4-OH-TAM-O-glucuronide and *trans*-endoxifen-O-glucuronide were determined by UPLC as indicated in the Methods. **Panel A**, from top to bottom, *trans*- and *cis*- 4-OH-TAM-O-glucuronide standards; cells incubated with media along; *trans*-4-OH-TAM-O-glucuronide in media before incubation; *trans*-4-OH-TAM-O-glucuronide in media after incubation. **Panel B**, from top to bottom, *trans*- and *cis*- endoxifen-O-glucuronide standards; cells incubated with media along; *trans*-endoxifen-O-glucuronide in media before incubation; *trans*-endoxifen-O-glucuronide in media after incubation.

Figure 5. Effect of TAM and TAM metabolites on PGR gene expression in MCF-7 cells. Cells were incubated with 1×10^{-10} M E₂ (**A, B**), or without E₂ (**C**), for 12 h at 37°C in 5% CO₂. Total RNA was extracted and PGR mRNA levels were determined by RT-PCR as described in the Methods. PGR mRNA levels were expressed as the fold-induction of PGR mRNA levels observed for untreated cells (media). **Panel A**, PGR gene expression in E₂-induced MCF-7 cells treated with TAM and 4-OH-TAM isomers or glucuronides; **panel B**, PGR gene expression in E₂-induced MCF-7 cells treated with endoxifen isomers or glucuronides; **panel C**, PGR gene expression in non-induced MCF-7 cells treated with TAM, 4-OH-TAM or endoxifen isomers or their glucuronides. The E₂ positive control, the media along and vehicle negative control are shown on all three panels. E₂, cells incubated with 1×10^{-10} M E₂; 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

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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 describes the concentration of TAM or TAM metabolite used in this experiment. The mean \pm standard error is shown for three experiments.

Figure 1

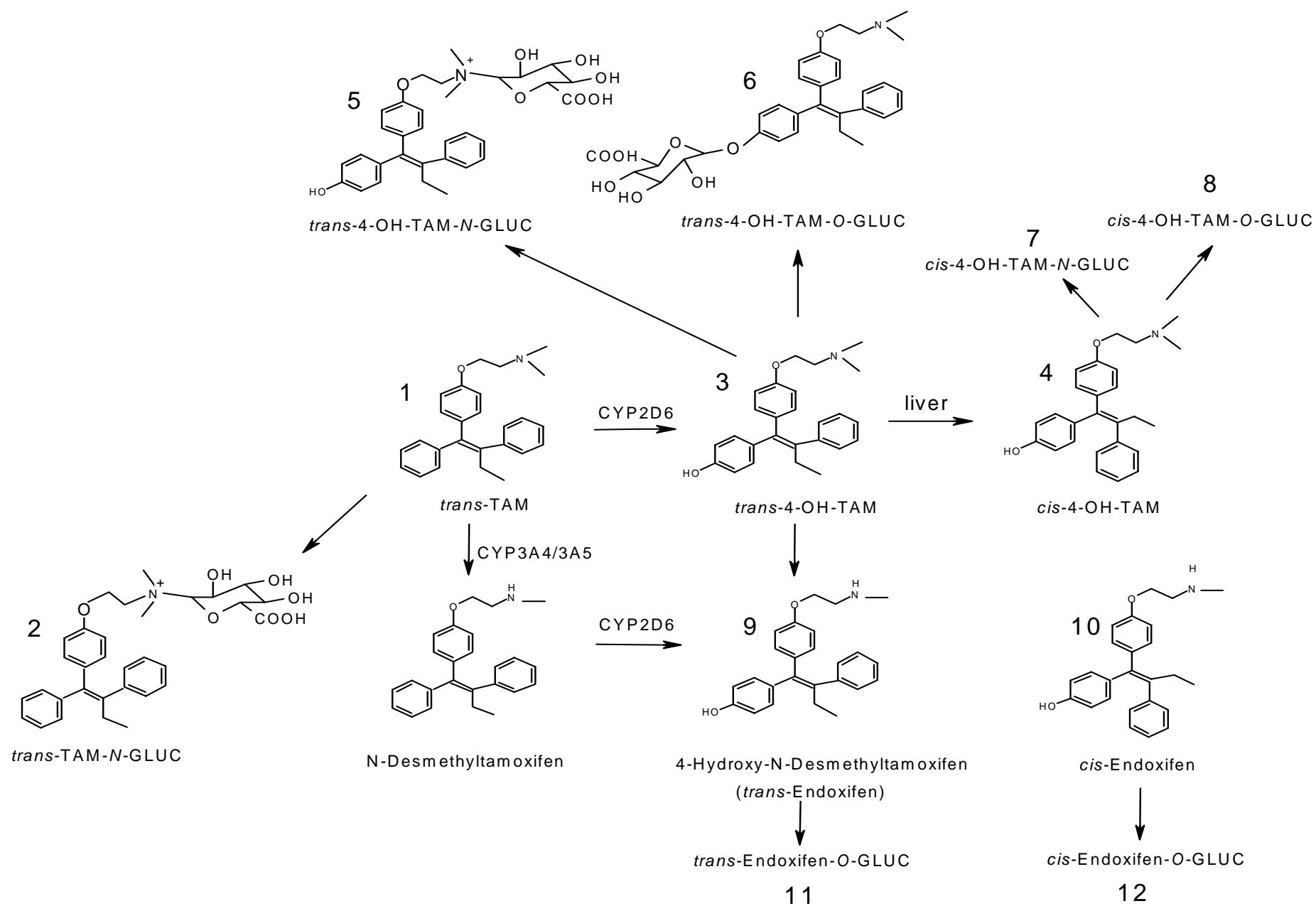


Figure 2A

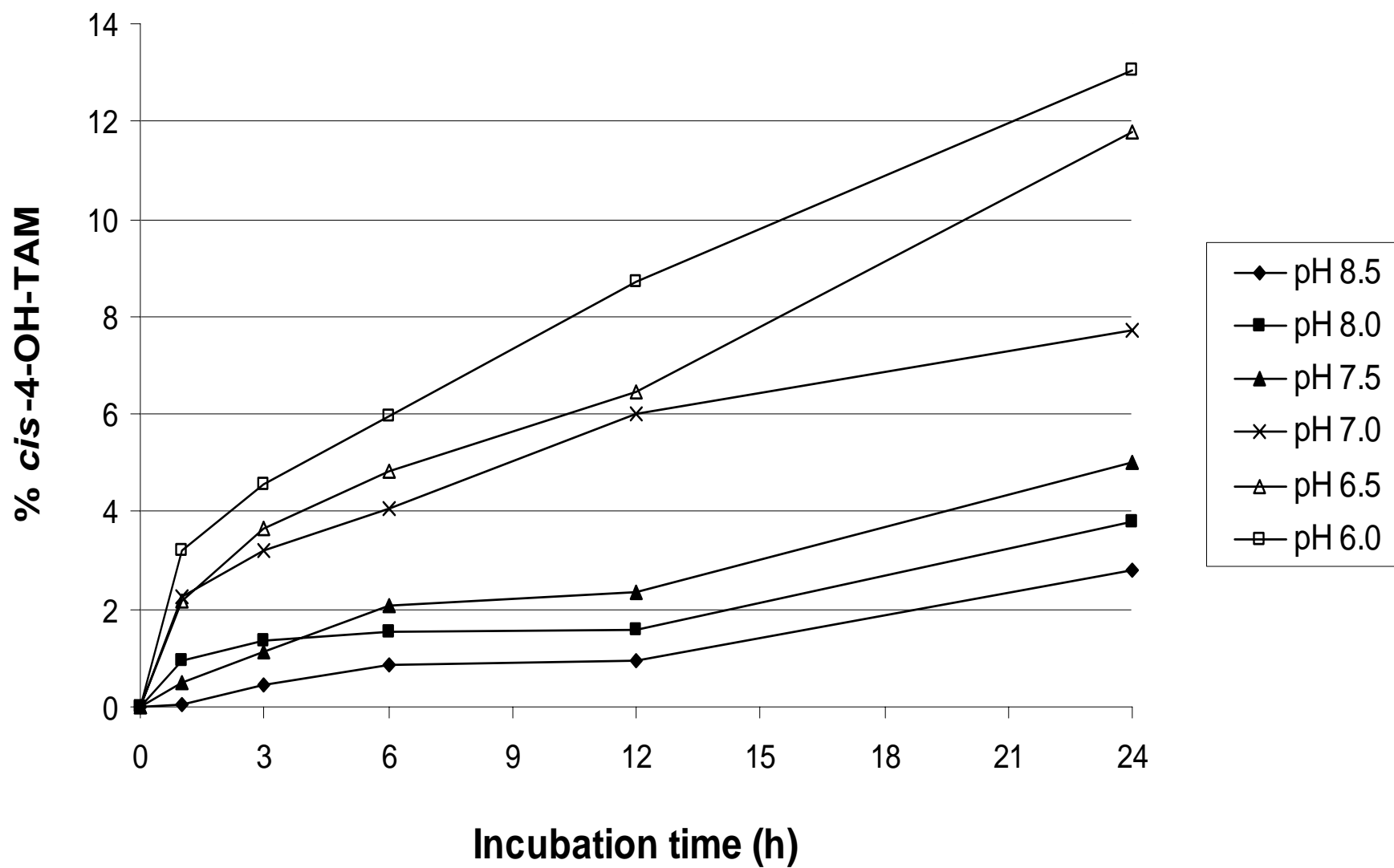


Figure 2B

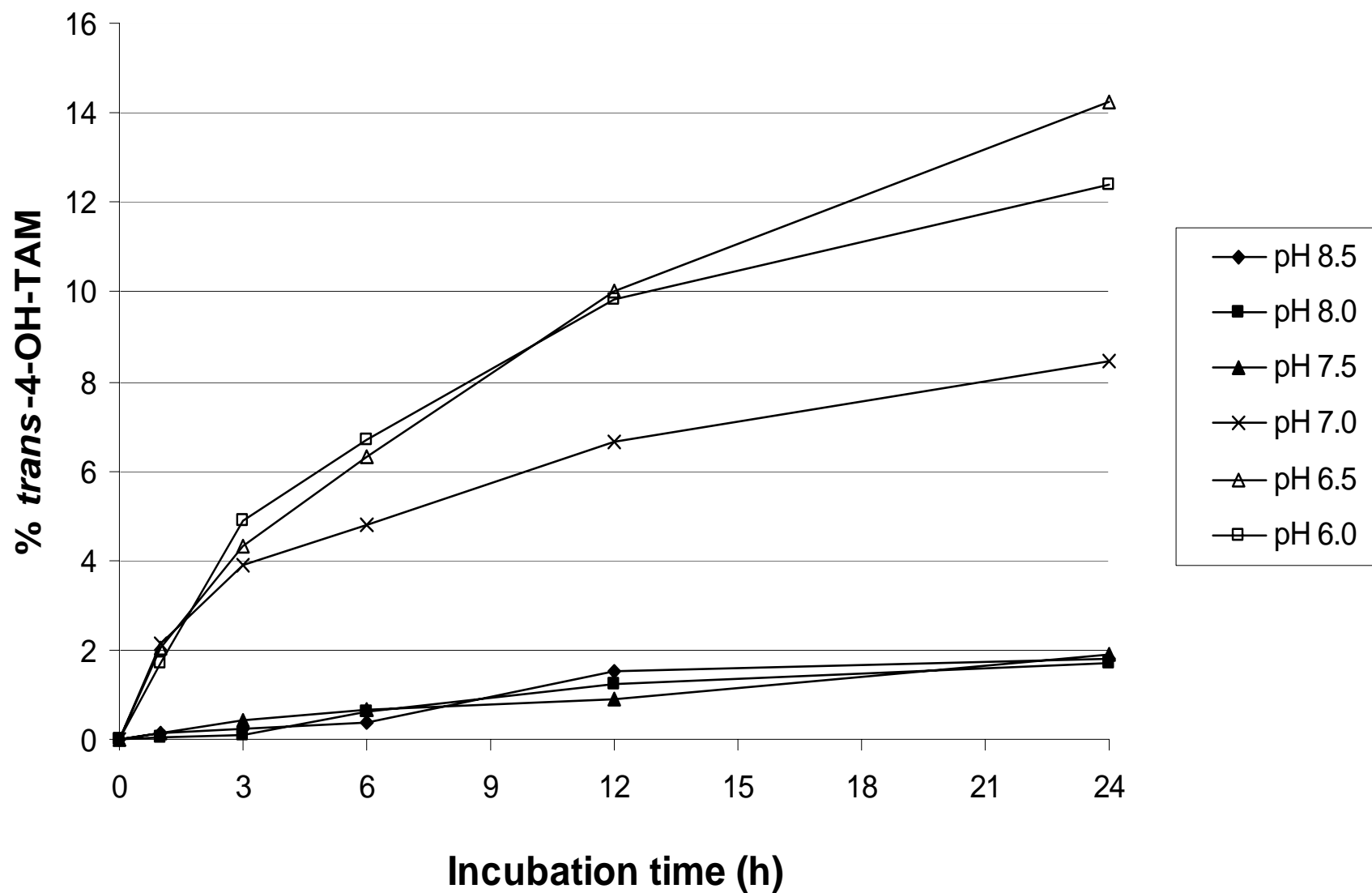


Figure 3

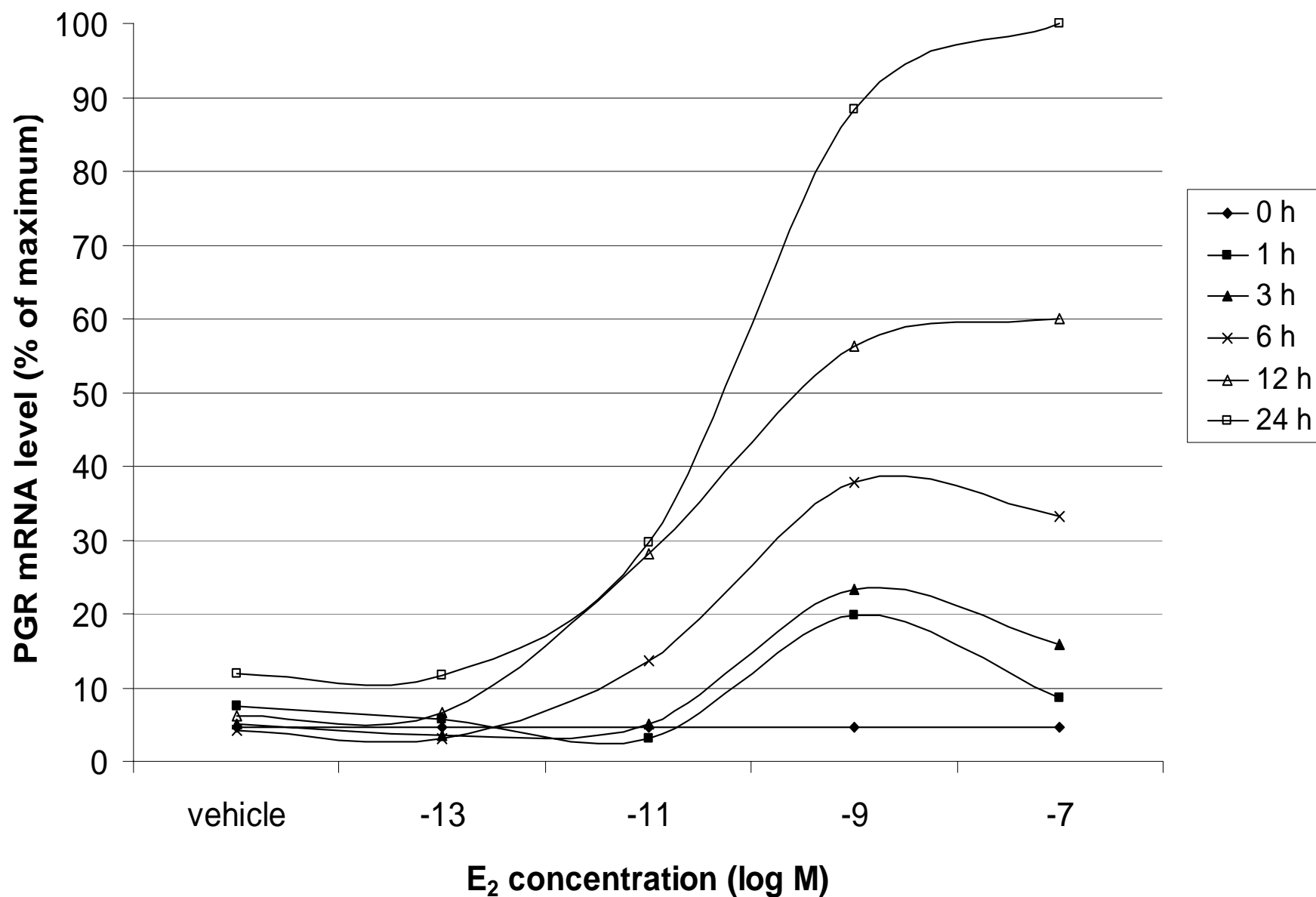


Figure 4A

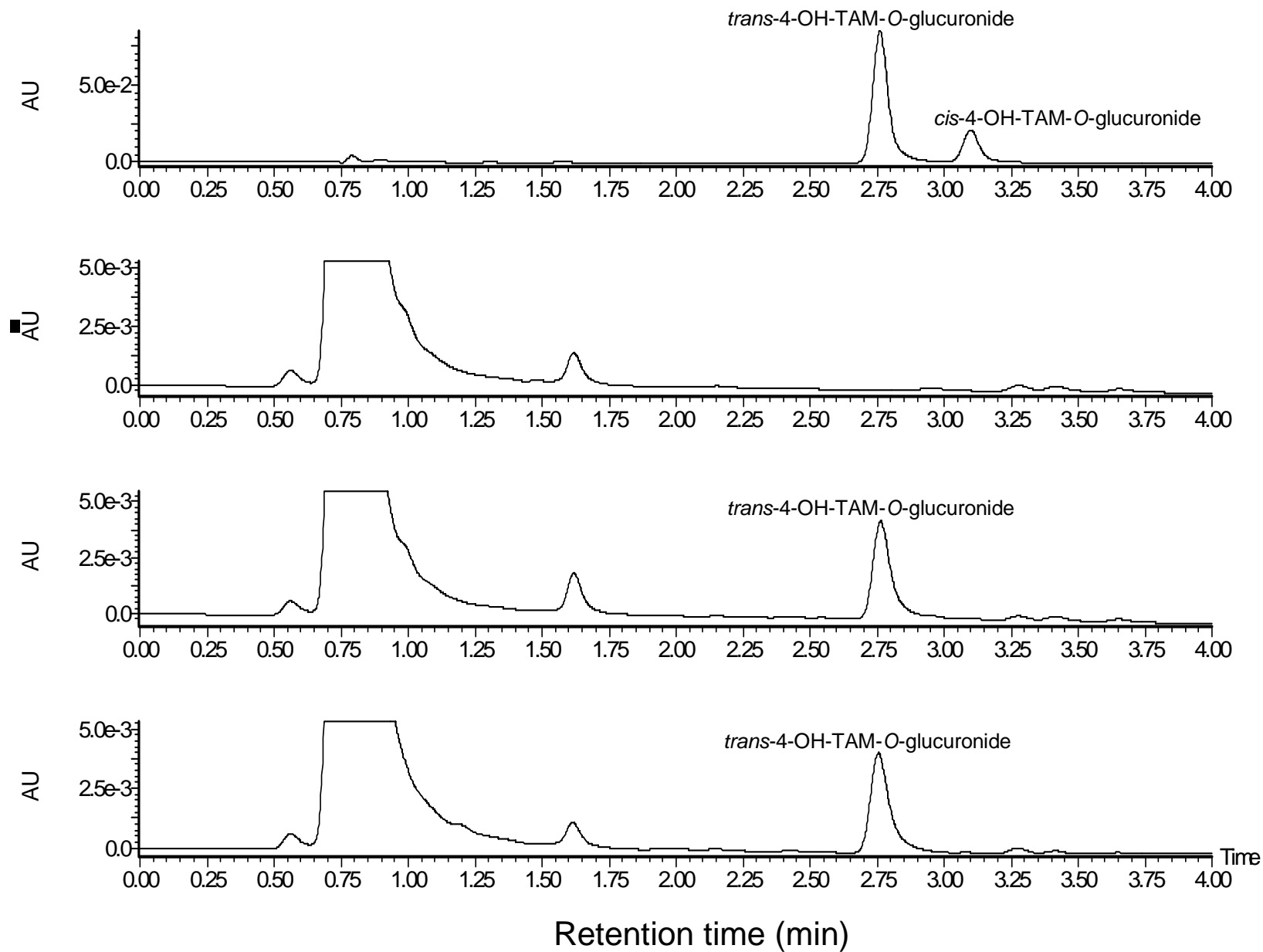


Figure 4B

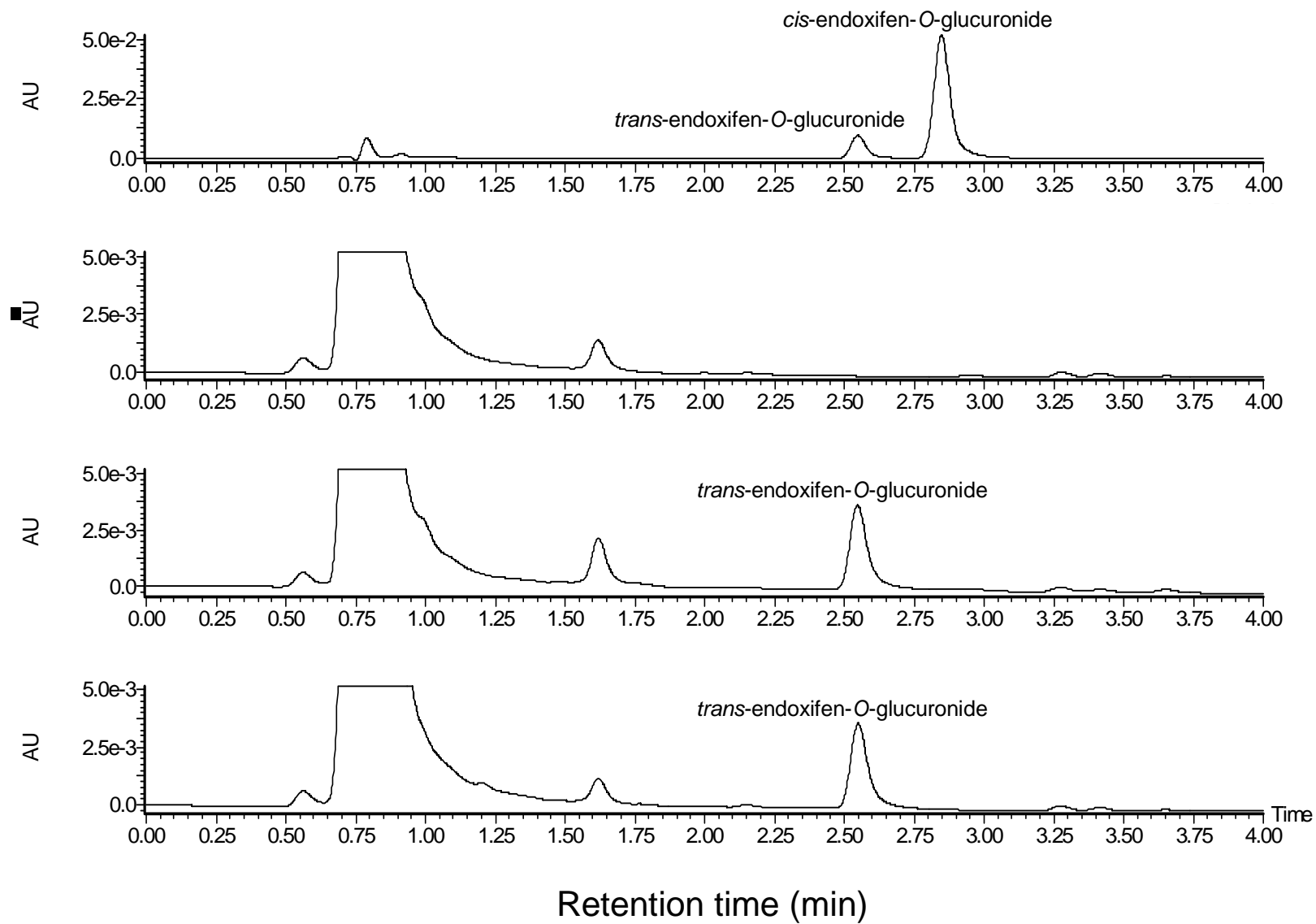


Figure 5A

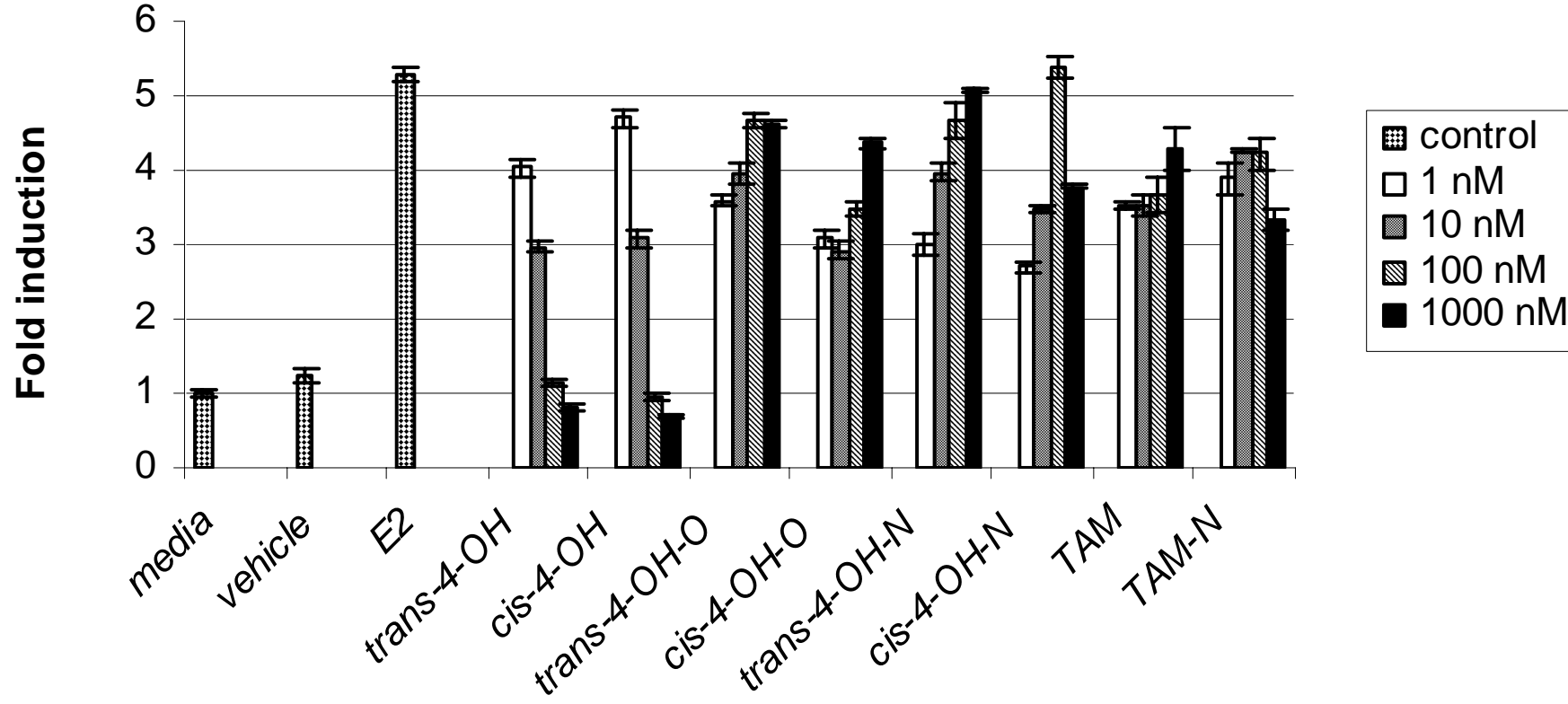


Figure 5B

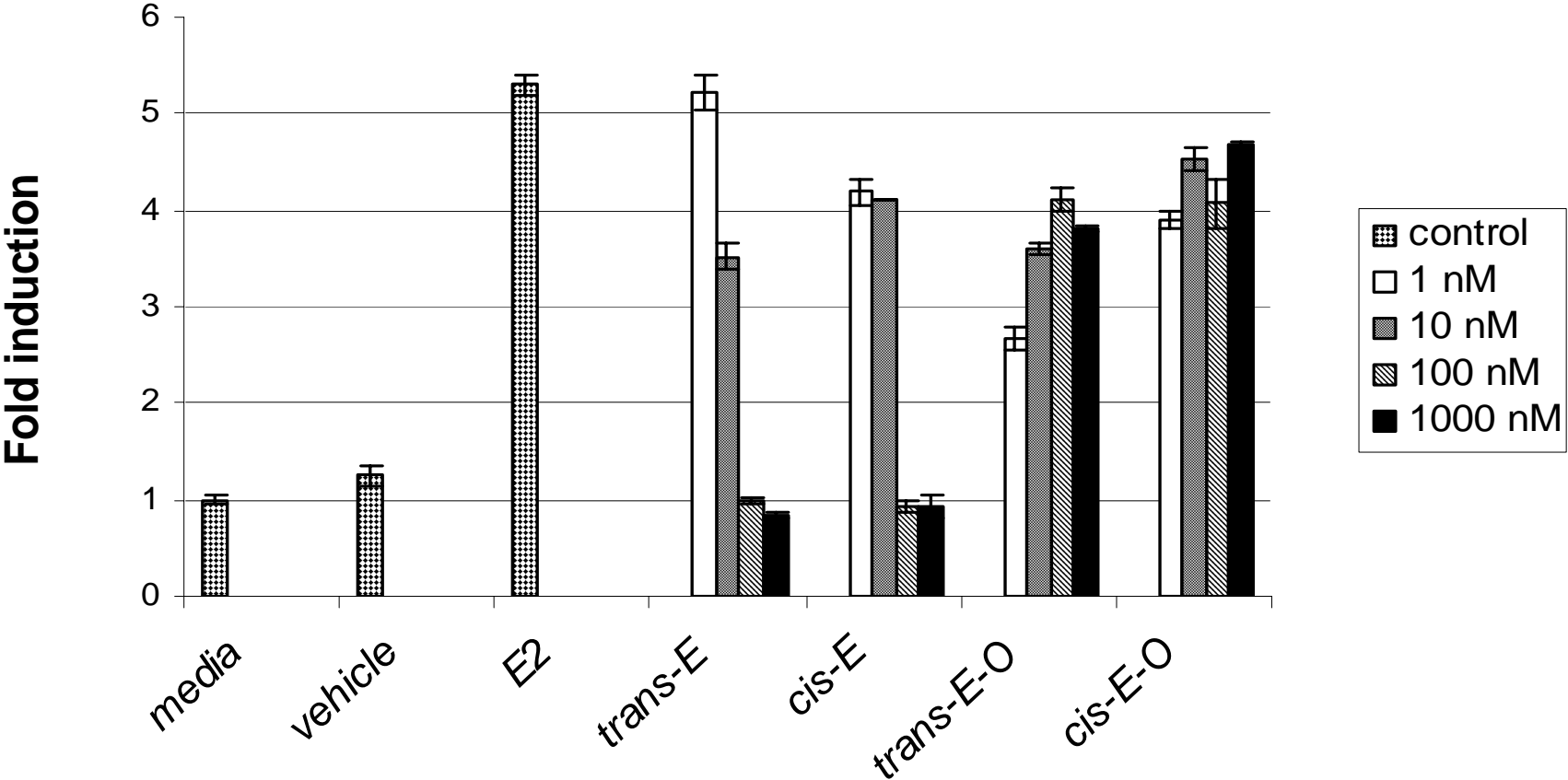


Figure 5C

