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Quaternary ammonium-linked glucuronidation of *trans*-4-hydroxytamoxifen, an active metabolite of tamoxifen, by human liver microsomes and UDP-glucuronosyltransferase 1A4

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Abbreviations:
BSA, bovine serum albumin
ESI-TOF-MS, electrospray ionization time-of-flight mass spectrometry
ER, estrogen receptor
GA, glucuronic acid
HRP, horseradish peroxidase
4-HO-TAM, 4-hydroxytamoxifen
HPLC, high performance liquid chromatography
metabolite Y, 1-[4-(2-hydroxyethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene)

ABSTRACT

Tamoxifen (TAM), a nonsteroidal antiestrogen, is the most widely used drug for chemotherapy of hormone-dependent breast cancer in women. *Trans*-4-hydroxy-TAM (*trans*-4-HO-TAM), one of the TAM metabolites in humans, has been considered to be an active metabolite of TAM because of its higher affinity toward estrogen receptors (ERs) than the parent drug and other side-chain metabolites. In the present study, we found a new potential metabolic pathway of *trans*-4-HO-TAM and its geometrical isomer, *cis*-4-HO-TAM, via *N*-linked glucuronic acid conjugation for excretion in humans. N^+-Glucuronides of 4-HO-TAM isomers were isolated along with *O*-glucuronides from a reaction mixture consisting of *trans*- or *cis*-4-HO-TAM and human liver microsomes fortified with UDP-glucuronic acid and identified with their respective synthetic specimens by high performance liquid chromatography–electrospray ionization time-of-flight mass spectrometry. Although *N*- and *O*-glucuronidating activities of human liver microsomes toward *trans*-4-HO-TAM were nearly comparable, *O*-glucuronidation was predominant for *cis*-4-HO-TAM conjugation. Only UGT1A4 catalyzed the *N*-linked glucuronidation of 4-HO-TAM among recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in insect cells. In contrast, all UGT isoforms, except for UGT1A3 and UGT1A4, catalyzed *O*-glucuronidation of 4-HO-TAM. Although *O*-glucuronidation of 4-HO-TAM greatly decreased binding affinity for human ERs, 4-HO-TAM N^+-glucuronide still had binding affinity similar to 4-HO-TAM itself, suggesting that N^+-glucuronide might contribute to the biological activity of TAM in vivo.

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TAM, tamoxifen
HFC, 7-hydroxy-4-(trifluoromethyl)coumarin
TLC, thin-layer chromatography
UDPGA, UDP-glucuronic acid
UGT, UDP-glucuronosyltransferase

1. Introduction

Tamoxifen (TAM, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene) is the class representative of a group of nonsteroidal triphenylethylene antiestrogens and has been widely used for the treatment of advanced breast cancer [1]. Also known is that TAM can reduce the risk of estrogen receptor (ER)-positive breast cancer as a chemopreventive agent in healthy women [2]. The antiestrogenic activity of TAM is based on competing activity with 17β-estradiol (E2) for ERs. TAM shows higher binding affinity to one isoform of human ER, ERα, than to another isoform, ERβ [3]. The metabolism and pharmacokinetics of TAM have been extensively studied in female patients and animals. In the human, orally administered TAM is converted to several metabolites, such as *N*-desmethyl-TAM, *trans*-4-hydroxy-TAM (*trans*-4-HO-TAM), *N*-desdimethyl-TAM, 4-hydroxy-*N*-desmethyl-TAM (4-HO-*N*-desmethyl-TAM), TAM *N*-oxide and the primary alcohol designated as metabolite Y [4–7].

Trans-4-HO-TAM has been considered to be an active metabolite of TAM because of its higher affinity toward ERs than the parent drug and other side-chain metabolites [8]. Interestingly, *trans*-4-HO-TAM was partially converted in vivo to its geometrical isomer, *cis*-4-HO-TAM, which may have weak estrogenic properties [9]. Our previous study on Phase II metabolism of 4-HO-TAM showed that the geometrical isomers of 4-HO-TAM were selectively glucuronidated to form *O*-glucuronide in the manner of *cis* ≫ *trans* by the UDP-glucuronosyltransferase (UGT) isoform UGT2B15 in human liver microsomes and sulfated in the manner of *trans* ≫ *cis* by the sulfotransferase isoform SULT1A1 in human liver cytosol [10].

Lien et al. [11] reported in an extensive study on the distribution of TAM and its metabolites in human biological fluids that bile and urine were rich in hydroxylated, Phase II conjugated metabolites (4-HO-TAM, 4-HO-*N*-desmethyl-TAM, and metabolite Y), whereas unconjugated 4-HO-TAM and unmetabolized TAM were the predominant species in feces. The existence of glucuronic acid conjugates was suggested by showing that treatment of the fecal extract from one patient with β-glucuronidase increased the concentration of TAM and TAM metabolites [11]. Also, entero-hepatic circulation of unmetabolized TAM as well as hydroxylated TAM metabolites in patients who were administered TAM was observed [12]. These findings indicated important effects of glucuronidation of TAM and its biologically active metabolites on the pharmacological activities of TAM.

Recently, we reported quaternary ammonium-linked glucuronidation of TAM in vitro to reveal a possible excretion pathway of TAM whereby TAM could be excreted into bile via TAM N^+-glucuronide [13]. Moreover, TAM N^+-glucuronide still had binding affinity similar to TAM itself for human estrogen receptors (ERs), ERα and ERβ, suggesting that TAM N^+-glucuronide might contribute to the biological activity of TAM in vivo. In contrast, in the case of 4-HO-TAM, *O*-glucuronidation greatly reduced the relative binding affinity of 4-HO-TAM for MCF-7 cytosolic ERs to 1/1000, which indicated that *O*-glucuronidation of 4-HO-TAM represented solely a deactivation pathway [14]. However, no information is available on *N*-glucuronidation of 4-HO-TAM.

In the present study, we investigated whether human liver microsomes and recombinant UGT isoforms were capable of catalyzing *N*-glucuronidation of 4-HO-TAM geometrical isomers to reveal a new potential excretion pathway of the active metabolite. Binding affinity of *trans*-4-HO-TAM N^+-glucuronide to human estrogen receptors, ERα and ERβ, was also investigated in comparison to that of *trans*-4-HO-TAM *O*-glucuronide.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), E2, eugenol, β-glucuronidase (type VII-A from *Escherichia coli*, 100 U/mL), TAM and UDP-glucuronic acid (UDPGA) were purchased from Sigma Chemicals Co. (St. Louis, MO). 4-HO-TAM obtained from Sigma was a mixture of *trans*- and *cis*-isomers. The isomeric mixture was separated by HPLC as reported previously [15,16], which provided *cis*- and *trans*-4-HO-TAMs with purity of more than 98 and 99%, respectively. The *cis*- and *trans*-HO-TAMs were eluted at retention times of 7.9 and 9.0 min, respectively, from the HPLC column and identified by ^{1}H-NMR spectroscopy with a Bruker DRX-500 spectrometer (500 MHz, Karlsruhe, Germany) as reported previously [17] and by mass spectrometry with a mass spectrometer model TSQ 700 (Finnigan MAT, San Jose, CA). 7-Hydroxy-4-(trifluoromethyl)coumarin (HFC) was purchased from Kanto Chemicals Co. (Tokyo, Japan). Alamethicin, trifluoperazine and [^{14}C]UDPGA (300 mCi/mmol) were purchased from ICN Pharmaceuticals, Inc. *Trans*- and *cis*-4-HO-TAM *O*-glucuronides were chemically synthesized as previously described [14]. Microsomes prepared from insect cells expressing recombinant human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 expressed in BTI-TN-5B1-4 cells (derived from *Trichoplusia ni*) were purchased from Gentest Co. (Woburn, MA). Pooled male New Zealand white rabbit microsomes were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

2.2. *High performance liquid chromatography–electrospray ionization time-of-flight mass spectrometry (HPLC–ESI-TOF-MS) conditions*

HPLC was performed with an HPLC system (Waters 2695, Waters, Milford, MA) using a reverse phase column (CAPCELL PAK C18 AQ, 2.0 mm × 150 mm, 5 μm particles) (Shiseido, Tokyo, Japan) at a flow rate of 0.25 mL/min. The mobile phase was composed of (A) acetonitrile and (B) 100 mM ammonium acetate buffer, pH 5.0. The elution was done with a linear gradient of 25–60% A over 40 min. The mass spectrometer used was a Micromass model LCT. For a positive ESI-TOF-MS spectra of synthetic *trans*- and *cis*-4-HO-TAM N^+-glucuronides, samples were dissolved in methanol and infused via a syringe pump at a flow rate of 1 μL/min into the ion source. The positive ion electrospray needle voltage was 2500 V.

2.3. *Chemical synthesis of 4-HO-TAM N^+-glucuronides*

The *trans*- and *cis*-4-HO-TAM N^+-glucuronides were synthesized based on a method reported previously for the synthesis of TAM N^+-glucuronide [13] as shown in Fig. 1. The 4-hydroxyl group of *trans*-4-HO-TAM (**1**) was protected by acetylation and sequentially reacted with methyl(2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide)uronate to form a quaternary linked glucuronide derivative (**2**). During the reaction, isomerization took place to form an approximate 3:4 mixture of *trans*- and *cis*-isomers. After the protective groups were hydrolyzed, the *trans*- (**3**) and *cis*- (**4**) N^+-glucuronides were separated by HPLC. Because of the isomerization, a similar result was obtained when a 1:1 mixture of *trans*- and *cis*-4-HO-TAM was used for the synthesis.

Trans-4-HO-TAM (50 mg, 0.13 mmol) was dissolved in 0.2 mL of acetic anhydride and stirred for 24 h at room temperature. After acetic anhydride was evaporated in vacuo at 40 °C, the residue was dissolved in 0.2 mL of dichloromethane containing methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate (80 mg, 0.2 mmol) and stirred for 24 h at room temperature. The organic solvent was removed by evaporation, and the residue was dissolved in 1 mL methanol. The methanolic solution was alkalinized with 0.2 mL of 0.5 M aqueous sodium bicarbonate and stirred for 12 h at room temperature to hydrolyze the protective acetyl groups and carboxyl methyl ester. After the mixture was adjusted with 0.1N HCl to pH 5.0, it was loaded onto an HPLC column (CAPCELL PAK C18 AQ, 4.6 mm × 150 mm). The column was eluted with methanol:0.1 M ammonium acetate, pH 5.0 (7:3, v/v), at a flow rate of 1 mL/min, with UV monitoring at 254 nm. *Trans*- and *cis*-4-HO-TAM N^+-glucuronides were eluted at retention times of 16.3 and 12.6 min, respectively. Evaporation of the eluate for the *trans*- and *cis*-N^+-glucuronides yielded 13.3 and 11.8 mg of white powder (18.1 and 16.1%), respectively. Noted for *trans*-4-HO-TAM N^+-glucuronide by ^{1}H-NMR (500 MHz, dimethyl sulfoxide-d6) were the following: δ 0.84 (t, 3H, *J* 7.4 Hz, CH_2CH_3), 2.40 (q, 2H, *J* 7.4 Hz, CH_2CH_3), 3.12 (s, 3H, N-CH_3), 3.16 (m, 1H, H-4′), 3.18 (s, 3H, N-CH_3), 3.27 (m, 1H, H-3′), 3.40 (d, 1H, *J* 9.5 Hz, H-5′), 3.53 (m, 1H, H-2′), 3.77–3.84 (m, 2H, N-CH_2CH_2-O), 4.38 (br.t, 2H, *J* 5.0 Hz, N-CH_2CH_2-O), 4.64 (d, 1H, *J* 8.9 Hz, H-1′), 5.50 (bs, 1H, OH-3′), 6.02 (bs, 1H, OH-2′), 6.57 (d, 2H, *J* 8.7 Hz, ArH, *ortho* to NCH_2CH_2O–), 6.74–7.20 (m, 11H, ArH), and 9.47 (s, 1H, COOH). Values for ESI-TOF-MS *m*/*z* (relative intensity) were 564.3326 $[M]^+$ (100%) and 388.2823 (18%). Calculated mass was 564.2597 for $C_{32}H_{38}NO_8$ $[M]^+$. Results for *cis*-4-HO-TAM N^+-glucuronide by ^{1}H-NMR (500 MHz, dimethyl sulfoxide-d6) were δ 0.84 (t, 3H, *J* 7.4 Hz, CH_2CH_3), 2.40 (q, 2H, *J* 7.4 Hz, CH_2CH_3), 3.15 (m, 1H, H-4′), 3.16 (s, 3H, N-CH_3), 3.24 (m, 1H, H-3′), 3.26 (s, 3H, N-CH_3), 3.45 (d, 1H, *J* 9.5 Hz, H-5′), 3.58 (m, 1H, H-2′), 3.86–3.95 (m, 2H, N-CH_2CH_2-O), 4.52 (br.t, 2H, *J* 5.0 Hz, N-CH_2CH_2-O), 4.72 (d, 1H, *J* 8.9 Hz, H-1′), 5.51 (bs, 1H, OH-3′), 6.08 (bs, 1H, OH-2′), 6.40 (d, 2H, *J* 8.6 Hz, ArH, *ortho* to NCH_2CH_2O–), 6.60 (d, 2H, *J* 8.6 Hz, ArH, meta to NCH_2CH_2O–),6.96–7.20 (m, 9H, ArH), and 9.20 (s, 1H, COOH). Values for ESI-TOF-MS *m*/*z* (relative intensity) were 564.3104 $[M]^+$ (100%) and 388.0900 (14%). Calculated mass was 564.2597 for $C_{32}H_{38}NO_8$ $[M]^+$.

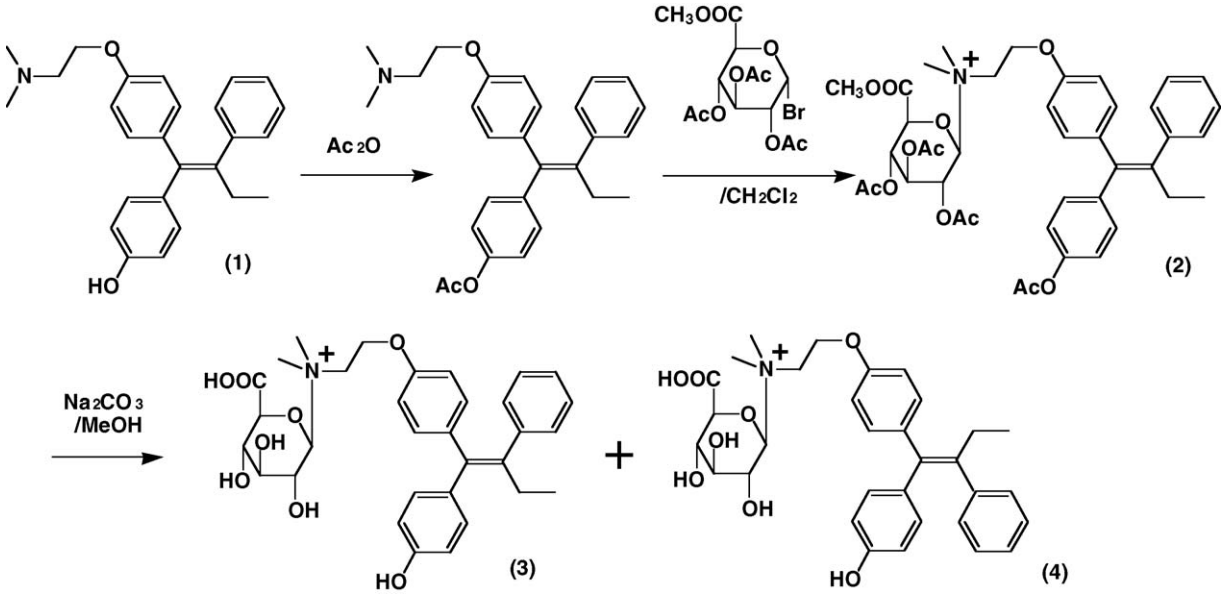

Fig. 1 – Chemical synthesis of *trans*-4-HO-TAM N^+-glucuronide.

2.4. NMR spectroscopy

The 500 MHz ^{1}H-NMR spectra were recorded on a Bruker model DRX500 at 300 K. Synthetic 4-HO-TAM N^{+}-glucuronides were dissolved in dimethyl sulfoxide-d6 to a concentration of 1 mg/0.5 mL.

2.5. Human liver samples

Liver samples from four cancer patients (two females, two males) were obtained from the National Cancer Center Hospital, Tokyo, Japan. Informed consent was obtained from each patient prior to study entry. The present study was approved by an ethics committee of the National Cancer Center Hospital. All patients had undergone partial hepatectomy to remove liver metastases of colon cancer. Pathologically and histologically normal liver samples used in the study were obtained from normal portions of removed tissue. All of the fresh samples were rapidly frozen in liquid nitrogen and stored at −80 °C before use.

2.6. Preparation of liver microsomes

Preparation of liver microsomes from the four human subjects and male and female Sprague–Dawley rats ($n = 3$ each), male ddy mice ($n = 3$), male cynomolgus monkeys ($n = 2$), male beagle dogs ($n = 2$), and male Hartley guinea pigs ($n = 3$) was performed by the following procedure. Approximately 1 g of liver was homogenized in 4 ml of 50 mM Tris–HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ [18]. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C, and the supernatant was collected. The supernatant was centrifuged at 105,000 × *g* for 1 h at 4 °C, and the resultant pellet was resuspended in 1 mL of the same buffer and used as the microsomal fraction. Protein concentrations of microsomal fractions were measured by the method of Bradford [19] using BSA as a standard.

2.7. Enzyme assay

Determinations of glucuronidating activity of human liver microsomes and insect cell microsomes containing expressed UGT isoforms toward trifluoperazine, eugenol, and HFC were carried out using [^{14}C]UDPGA as described previously [13]. Human liver microsomes were activated with alamethicin (50 μg/mg protein) for 15 min in an ice bath as described by Fisher et al. [20]. This step was omitted for the insect cell microsomes because alamethicin did not affect glucuronidating activity of insect microsomes. The radioactivity of the glucuronide was measured by radioluminography with a BAS 2000 bioimaging analyzer (Fuji Photo Films Co., Ltd., Tokyo, Japan).

The *N*- and *O*-glucuronidating activities of human and insect microsomes toward *trans*- and *cis*-4-HO-TAMs were determined by HPLC-UV analysis. The reaction was performed for 1 h at 37 °C in a mixture consisting of 50 mM Tris–HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, microsomal protein (50 μg) and 2 mM UDPGA in a final volume of 50 μL. The reaction was started by the addition of the substrate which was dissolved in a solution (5 μL) consisting of ethanol and buffer (1:1, v/v) and terminated by the addition of 50 μL of methanol containing 1 μM TAM as an internal standard. After centrifugation at 12,000 × *g* for 5 min, 5 μL of the supernatant was analyzed by HPLC with UV 286 nm detection using a reverse phase column (CAPCELL PAK C18 AQ, 2 mm × 150 mm, 5 μm particles, Shiseido) at a flow rate of 0.25 mL/min. Elution was done with a 25–60% (v/v) linear gradient of acetonitrile over 40 min in 100 mM ammonium acetate buffer (pH 5.0). Under these conditions, *O*-glucuronides of *trans*- and *cis*-4-HO-TAMs were eluted at retention times of 12.8 and 14.0 min, N^{+}-glucuronides of *cis*- and *trans*-4-HO-TAMs at retention times of 21.5 and 22.4 min, and *cis*- and *trans*-4-HO-TAM retention times of 26.9 and 27.2 min, respectively; that of the internal standard TAM was 38.2 min. Determinations of 4-HO-TAM *N*-glucuronidating activity of liver microsomes from experimental animals were performed under the same reaction conditions as described above. Data were expressed as the arithmetic mean values ± S.D. obtained from at least three replicated incubations. For determination of apparent kinetic constants for *N*-glucuronidation of 4-HO-TAM, substrate concentrations ranging from 5 to 200 μM were used. The kinetic constants were determined by extrapolation from Michaelis–Menten plots.

2.8. Identification of trans- and cis-4-HO-TAM N^{+}-glucuronides formed by human liver microsomes in the presence of UDPGA

For identification of 4-HO-TAM N^{+}-glucuronides formed by human liver microsomes, the reaction was performed as indicated above. After termination, the supernatant was lyophilized, and the residue was dissolved with 20 μL methanol for analysis by HPLC–ESI-TOF-MS. For treatment with β-glucuronidase, chromatographic fractions containing N^{+}-glucuronide were pooled, and the solvent was evaporated to dryness in vacuo at 40 °C. The residue obtained was dissolved in 100 μL of 4 mM sodium phosphate buffer (pH 6.8) containing 10 units of β-glucuronidase and incubated for 1 h at 37 °C. The incubation mixture was filtered through a disc filter (Kanto) and subjected to HPLC performed under the same conditions as described above.

2.9. Estrogen receptor binding

The competitive binding affinities of *trans*-4-HO-TAM, *trans*-4-HO-TAM N^{+}- and *O*-glucuronides, and diethylstilbestrol (DES) to E2 for human ERα and ERβ were assayed using the Ligand Screening System kit by TOYOBO (TOYOBO Co., Ltd., Osaka, Japan). Briefly, purified recombinant human ERα or ERβ was incubated in a microplate with various concentrations of these ligands in the presence of E2 (12.5 nM) at 4 °C for 1 h. After incubation, unbound E2 was allowed to compete with anti-E2 antibody and horseradish peroxidase (HRP)-labeled E2 at 4 °C for 1 h. After washing the plate, the remaining peroxidase-labeled E2 bound on the well was measured by densitometry using the microplate reader Model SAFIRE (Tecan Japan Co., Ltd., Tokyo, Japan) at 450 nm according to the manufacturer's instructions. The relative binding affinity of each competitor is taken at the ratio of IC_{50} values (concentration of ligand required to reduce the specific E2 binding by 50%) to that of

DES. Data were expressed as arithmetic mean values ± S.D. obtained from at least three replicated assays.

3. Results

3.1. Chemical synthesis of 4-HO-TAM N^+-glucuronides

After protection of the 4-hydroxyl group, a reaction of 4-acetoxy-TAM with methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate in dichloromethane gave the *N*-linked glucuronides. NMR spectra of the *trans*- and *cis*-glucuronides showed the signal for the anomeric proton on the sugar ring at δ 4.64 and 4.72 ppm, respectively, as a doublet with a coupling constant of 8.9 Hz for both isomers. These chemical shifts and coupling constants were characteristic of N^+-linked-β-glucuronides [21–23]. Separation of *N*,*N*-dimethyl proton signals as singlets at δ 3.11 and 3.18 ppm for the *trans*-isomer and δ 3.19 and 3.26 ppm for the *cis*-isomer indicated that each of the methyl groups was unequivalent due to the formation of the fourth N–C bond.

3.2. Identification of 4-HO-TAM N^+-glucuronides formed by human liver microsomes

Enzymatic formation of 4-HO-TAM N^+-glucuronides by human liver microsomes was first characterized by HPLC (Fig. 2) and HPLC–ESI-TOF-MS (Fig. 3). Fig. 2 shows representative HPLC chromatograms of enzymatically formed *O*- and N^+-glucuronides from *trans*- and *cis*-4-HO-TAMs by human microsomes in the presence of UDPGA. *O*- and N^+-glucuronides of *trans*-4-HO-TAM were formed at similar rates by liver microsomes with retention times of 12.8 and 22.4 min (chromatogram B), respectively, which were identical to those of the respective synthetic specimens. *O*- and N^+-glucuronides from the *cis*-isomer eluted at 14.0 and 21.5 min, respectively, and were identified with the synthetic specimens (chromatogram D). In contrast to the *trans*-isomer, predominant formation of *O*-glucuronide was observed from *cis*-4-HO-TAM whereas a lesser amount of N^+-glucuronide was formed. Treatment with β-glucuronidase of the N^+-glucuronides eluted from the HPLC column afforded corresponding isomers of 4-HO-TAM, which were identified by HPLC-MS (data not shown). MS analysis of the eluted 4-HO-TAM N^+-glucuronide isomers showed mass spectra (Fig. 3) very similar to those of the respective synthetic specimens. The mass spectrum for the *trans*-isomer showed an $[M^+]$ ion at *m*/*z* 564.3213 and a fragment ion at *m*/*z* 388.2580, corresponding to the parent drug 4-HO-TAM + H with loss of the glucuronic acid moiety (176 atomic mass units). The *cis*-isomer gave a similar spectrum with an $[M^+]$ ion at *m*/*z* 564.3438 and fragment ions at *m*/*z* 388.2455 and 337.0770.

3.3. Glucuronidation of 4-HO-TAM isomers by human liver microsomes

The rates of *O*- and *N*-linked glucuronide formation from 4-HO-TAM isomers were determined using liver microsomes from 4 donors (two females, F1 and F2; two males, M1 and M2). As shown in Table 1, there was a large difference in the ratio of *O*-glucuronidation to *N*-glucuronidation between *trans*- and

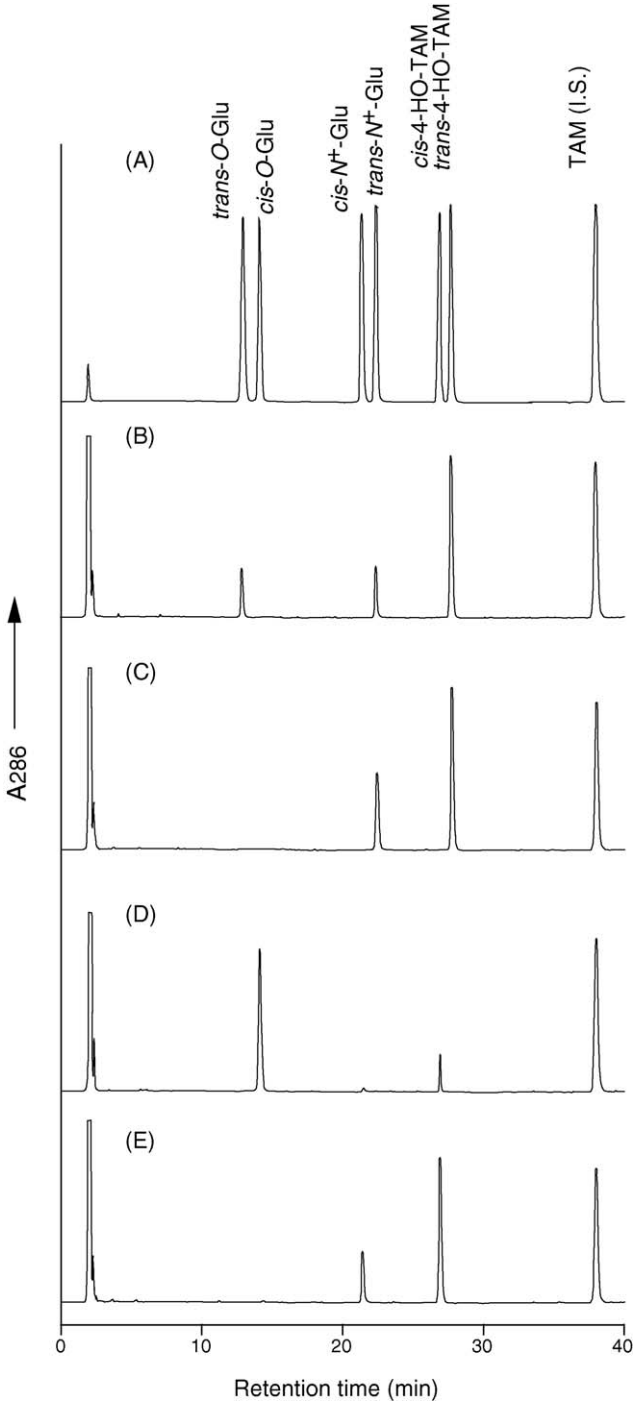

Fig. 2 – HPLC separation of *O*- and N^+-glucuronides of *trans*- and *cis*-4-HO-TAMs. (A) Synthetic specimens of *O*-glucuronides of *trans*- (*trans*-*O*-Glu) and *cis*- (*cis*-*O*-Glu) 4-HO-TAMs and N^+-glucuronides of *trans*- (*trans*-N^+-Glu) and *cis*- (*cis*-N^+-Glu) 4-HO-TAMs. *Trans*- and *cis*-4-HO-TAMs and TAM as an internal standard are also shown. Human liver microsomes (from donor F4 in Table 1) (B and D) or recombinant UGT1A4 (C and E) were incubated at 37 °C for 2 h with 0.4 mM *trans*-4-HO-TAM (B and C) or *cis*-4-HO-TAM (D and E) in the presence of 2 mM UDPGA. Details are described in the text.

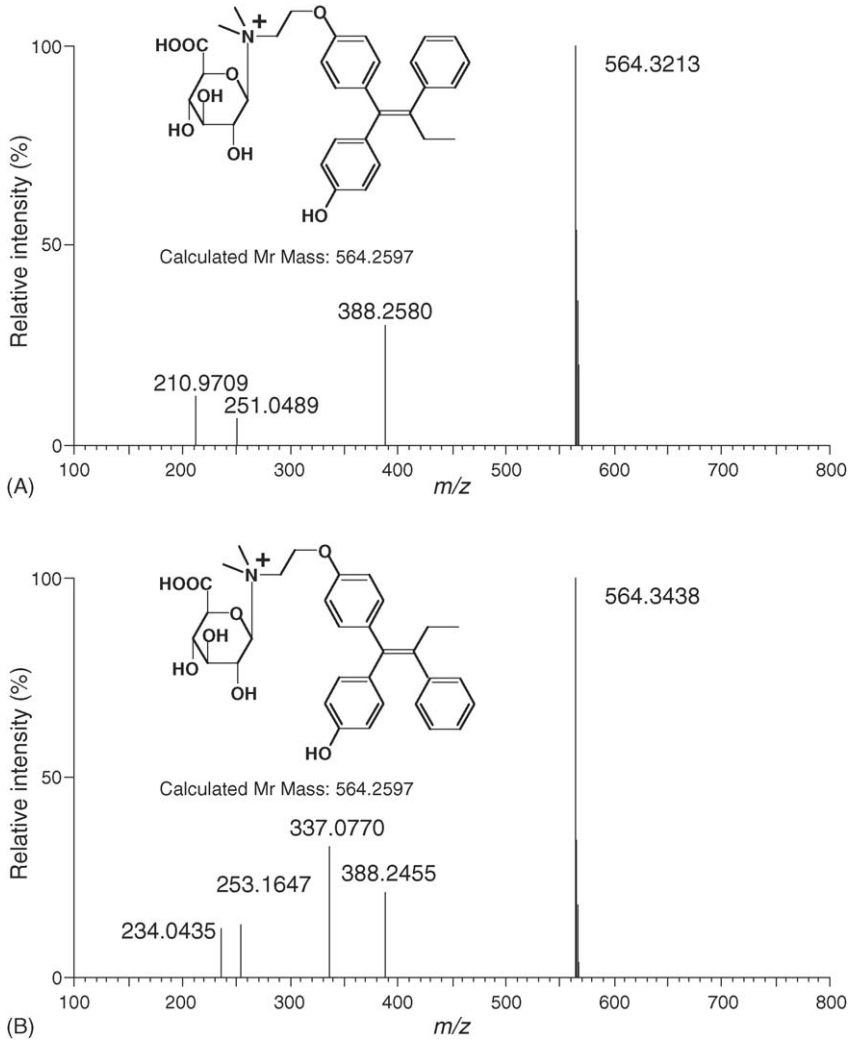

Fig. 3 – Representative mass spectra of *trans*- (A) and *cis*- (B) 4-HO-TAM N^+-glucuronides formed from 4-HO-TAM isomers by human liver microsomes. Spectra were taken at the retention times of 22.4 and 21.5 min in Fig. 2, panels B and D, respectively.

Table 1 – *N*- and *O*-Glucuronidation of *trans*- and *cis*-4-HO-TAM by human liver microsomes

Donor	Age	Specific activities (pmol/(min mg) protein)			
		Trans-4-HO-TAM		*Cis*-4-HO-TAM	
		O-Glu[a]	*N*-Glu	*O*-Glu	*N*-Glu
25 μM substrate					
F1	65	126.5 ± 4.5	106.7 ± 5.8	1816 ± 1.3	16.7 ± 2.2
F2	71	116.2 ± 15.8	85.8 ± 8.2	1950 ± 1.9	13.2 ± 3.1
M1	56	152.3 ± 2.1	91.0 ± 4.1	2148 ± 1.1	Trace
M2	67	146.0 ± 1.8	89.3 ± 2.5	2011 ± 1.5	11.3 ± 1.8
100 μM substrate					
F1	65	223.7 ± 4.5	301.6 ± 5.8	2494 ± 131	78.3 ± 1.3
F2	71	126.9 ± 8.1	185.1 ± 9.2	2038 ± 182	69.6 ± 3.9
M1	56	155.5 ± 2.1	216.0 ± 4.1	5256 ± 311	46.9 ± 1.3
M2	67	201.0 ± 1.8	223.7 ± 2.5	3899 ± 215	57.6 ± 1.3

Trans- or *cis*-4-HO-TAM (25 or 100 μM) was incubated for 1 h with human liver microsomes from two females (F1 and F2) and two males (M1 and M2) in the presence of 2 mM UDPGA in a final volume of 50 μL of 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. Activities for glucuronide formation were determined by HPLC as described in the text. Data are expressed as the arithmetic mean values ± S.D. of at least three experiments.

[a] *O*-Glu and *N*-Glu represent *O*- and *N*-glucuronide formations from *trans*- and *cis*-4-HO-TAMs, respectively.

Table 2 – *N*- and *O*-Glucuronidation of *trans*- and *cis*-4-HO-TAMs by human recombinant UGT isoforms

Isoform	Specific activities (pmol/(min mg) protein)				
	Trans-4-HO-TAM		*Cis*-4-HO-TAM		HFC
	O-Glu[a]	*N*-Glu	*O*-Glu	*N*-Glu	
UGT1A1	9.8 ± 3.4	N.D.[b]	14.8 ± 3.8	N.D.	443.3 ± 32.0
UGT1A3	23.0 ± 15.8	N.D.	42.1 ± 8.8	N.D.	922.9 ± 86.4
UGT1A4	N.D.	132.9 ± 10.6	N.D.	43.5 ± 9.3	N.D. (504.3 ± 10.6)[c]
UGT1A6	N.D.	N.D.	N.D.	N.D.	5882 ± 168
UGT1A7	N.D.	N.D.	N.D.	N.D.	2658 ± 108
UGT1A8	44.2 ± 17.1	N.D.	11.9 ± 5.9	N.D.	1967 ± 383
UGT1A9	10.7 ± 4.6	N.D.	49.3 ± 10.4	N.D.	6169 ± 111
UGT1A10	N.D.	N.D.	N.D.	N.D.	559.3 ± 190
UGT2B4	N.D.	N.D.	N.D.	N.D.	320.8 ± 40.4
UGT2B7	110.4 ± 32.8	N.D.	101.0 ± 38.9	N.D.	2247 ± 177
UGT2B15	18.3 ± 11.7	N.D.	374.1 ± 65.4	N.D.	2180 ± 569
UGT2B17	N.D.	N.D.	N.D.	N.D.	23.6 ± 14.5 (471 ± 120)[d]

Trans- or *cis*-4-HO-TAMs (25 μM) was incubated for 1 h with insect cell microsomes expressing each UGT isoform in the presence of 2 mM [^{14}C]UDPGA in a final volume of 50 μL of 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. HFC (50 μM) was used as a standard substrate for determining microsomal activity under the same conditions as stated above except for incubation time (30 min) for HFC. Activities toward trifluoperazine (200 μM) and eugenol (200 μM) were also determined as the indicated substrates for UGT1A4 and UGT2B17, respectively, with the incubation time of 30 min. Radioactive glucuronides formed were determined by TLC-radioluminography as described in the text. Data are expressed as the arithmetic mean values ± S.D. of at least three experiments.

[a] *O*-Glu and *N*-Glu represent *O*- and *N*-glucuronidating activities.

[b] N.D.: not detectable (less than 5 pmol/(min mg) protein).

[c] Activity toward trifluoperazine.

[d] Activity toward eugenol.

cis-4-HO-TAMs. Although *N*- and *O*-glucuronidating activities toward *trans*-4-HO-TAM were nearly comparable, *O*-glucuronidation was predominant for *cis*-4-HO-TAM conjugation. Although a small or a trace amount of N^+-glucuronide was formed from 25 μM *cis*-4-HO-TAM by microsomes from all subjects, a measurable amount of N^+-glucuronide was formed from the 100 μM substrate. In comparison of *O*-glucuronidation between *trans*- and *cis*-isomers, *cis*-4-HO-TAM was a much better substrate than the *trans*-isomer.

3.4. *Glucuronidation of 4-HO-TAM isomers by human UGT isoforms expressed in insect cells*

Recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) independently expressed in insect cells were examined for their ability to catalyze glucuronidation of 4-HO-TAM isomers (Table 2). Of these recombinant human enzymes, only UGT1A4 showed *N*-

Table 3 – Species difference in *N*- and *O*-glucuronidation of *trans*- and *cis*-4-HO-TAM

Species	Specific activities (pmol/(min mg) protein)				
	Trans-4-HO-TAM		*Cis*-4-HO-TAM		HFC[a]
	O-Glu[b]	*N*-Glu	*O*-Glu	*N*-Glu	
Human	126.9 ± 8.1	185.1 ± 16.2	2038 ± 212	69.6 ± 3.9	7.7 ± 0.8
Monkey (male)	196.7 ± 9.8	N.D.[c]	2308 ± 322	N.D.[c]	31.9 ± 14.3
Rat (male)	448.3 ± 5.8	N.D.	1538 ± 179	N.D.	4.7 ± 1.3
Rat (female)	300.0 ± 17	N.D.	1730 ± 209	N.D.	3.2 ± 0.9
Mouse (male)	411.7 ± 31	N.D.	1613 ± 156	N.D.	15.7 ± 2.0
Dog (male)	888.3 ± 55	N.D.	1860 ± 253	N.D.	34.0 ± 7.6
Guinea pig (male)	4183 ± 126	N.D.	3418 ± 332	N.D.	28.4 ± 4.0
Rabbit (male)	7518 ± 352	110.0 ± 3.4	4127 ± 284	73.5 ± 5.7	11.7 ± 2.2

Trans- or *cis*-4-HO-TAM (100 μM) was incubated for 1 h with hepatic microsomes from human (F1) and various species in the presence of 2 mM UDPGA in a final volume of 50 μL of 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. HFC (50 μM) was used as a standard substrate for determining microsomal activity under the same conditions as stated above except for incubation time (30 min). Activities for glucuronide formation were determined by HPLC as described in the text. Data are expressed as the arithmetic mean values ± S.D. of at least three experiments.

[a] nmol/(min mg) protein.

[b] *O*-Glu and *N*-Glu represent *O*- and *N*-glucuronide formations from *trans*- and *cis*-4-HO-TAMs, respectively.

[c] N.D., not detectable (less than 5 pmol/(min mg) protein).

glucuronidating activity toward 4-HO-TAM isomers. In contrast, various UGT isoforms, UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT2B7, and UGT2B15, exhibited *O*-glucuronidating activity toward 4-HO-TAM isomers. Among these UGTs, UGT2B15 had the highest *cis*-selective *O*-glucuronidating activity.

3.5. Species differences in liver microsomal glucuronidation of 4-HO-TAM

Rat, mouse, guinea pig, dog, and monkey liver microsomes had no detectable activity for *N*-glucuronidation of 4-HO-TAM isomers whereas they were shown to have HFC-glucuronidating activity (Table 3). Only human and rabbit microsomes had *N*-glucuronidating activity toward 4-HO-TAM. All animals, as well as humans, were shown to have *O*-glucuronidating activity toward both isomers of 4-HO-TAM. Interestingly, although human, monkey, rat, mouse, and dog liver microsomes had *cis*-selective *O*-glucuronidating activity toward 4-HO-TAM, guinea pig and rabbit liver microsomes had higher activity toward *trans*-4-HO-TAM than toward the *cis*-isomer.

3.6. Kinetic parameters for 4-HO-TAM N-glucuronidation by human liver microsomes and recombinant UGT1A4

Kinetic analysis of 4-HO-TAM *N*-glucuronidation was performed using human liver microsomes (from donor F1) and recombinant UGT1A4. Observed apparent K_m and V_{max} values for microsomal *trans*-4-HO-TAM *N*-glucuronidation were 56.2 ± 9.1 μM and 233.8 ± 31.8 pmol/(min mg) protein, respectively. However, because of the relatively high activity for *O*-glucuronidation of *cis*-4-HO-TAM, it was difficult to determine kinetic parameters for *N*-glucuronidation of *cis*-4-HO-TAM by human liver microsomes. However, these kinetic parameters for *cis*-HO-TAM *N*-glucuronidation could be determined by recombinant UGT1A4 due to the absence of *O*-glucuronidating activity in insect cell microsomes. An apparent K_m value similar to that with human microsomes was observed for *trans*-4-HO-TAM *N*-glucuronidation by recombinant UGT1A4 (41.5 ± 8.7 μM) whereas a relatively higher K_m value was observed for *N*-glucuronidation of the *cis*-isomer (168.9 ± 14.3 μM). Recombinant UGT1A4 catalyzed *trans*- and *cis*-4-HO-TAM *N*-glucuronidation with similar V_{max} values of 2121 ± 420.0 and 2520 ± 227.0 pmol/(min mg) protein, respectively.

3.7. Binding affinity of trans-4-HO-TAM glucuronides for human ERs

Binding affinities of *trans*-4-HO-TAM and its N^+- and *O*-glucuronides for human ERα and ERβ were determined by competitive binding analysis in comparison with that of DES. Based on the concentrations at which binding between E2 and ERs is reduced to 50% of binding in the absence of a competing ligand, *trans*-4-HO-TAM and its N^+-glucuronide had similar affinities for both ERs (Fig. 4). In addition, the synthetic *trans*-4-HO-TAM N^+-glucuronide was stable during the binding assays. Among the chemicals tested, DES competed with E2 at the lowest IC_{50} values of 25.8 ± 3.0 and 21.9 ± 2.8 nM for human ERα and ERβ binding, respectively. *Trans*-4-HO-TAM and *trans*-4-HO-TAM N^+-glucuronide competed at similar concentrations of 43.9 ± 5.2 and 42.8 ± 3.4 nM for ERα and 39.6 ± 5.1 and 35.4 ± 4.1 nM for ERβ, respectively. However, *O*-glucuronide of *trans*-4-HO-TAM showed low affinity for ERα and ERβ with IC_{50} values of 645.5 ± 21.3 and 509.5 ± 33.2, respectively. The relative binding affinities of DES, *trans*-4-HO-TAM, *trans*-4-HO-TAM N^+-glucuronide, and *trans*-4-HO-TAM *O*-glucuronide, taken at the ratio of IC_{50} values to that of DES, were 100, 58.8, 65.2, and 4.0 for ERα and 100, 51.2, 61.9, and 4.3 for ERβ, respectively.

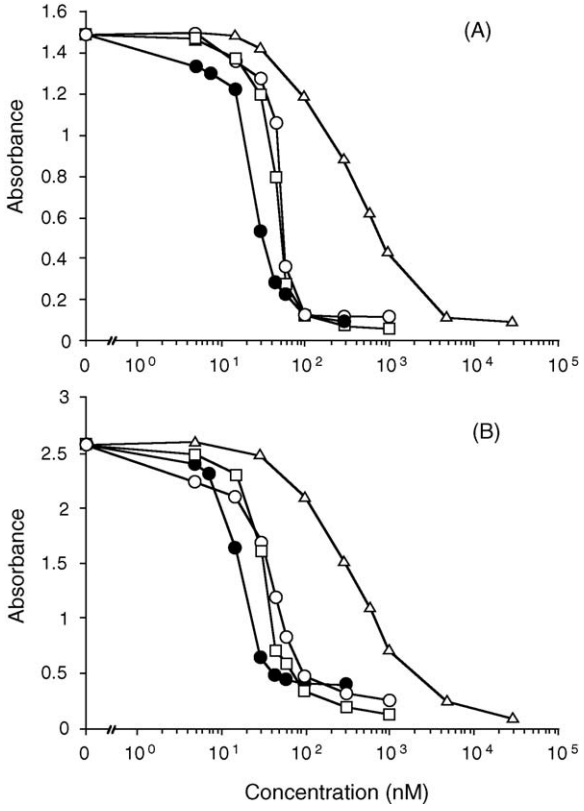

Fig. 4 – Competitive binding affinities of DES, *trans*-4-HO-TAM, and *trans*-4-HO-TAM N^+- and *O*-glucuronides for ERα and ERβ. Purified recombinant human ERα (panel A) or ERβ (panel B) was incubated with various concentrations of DES (●), *trans*-4-HO-TAM (□), *trans*-4-HO-TAM N^+-glucuronide (○), and *trans*-4-HO-TAM *O*-glucuronide (△) in the presence of E2 (12.5 nM) at 4 °C for 1 h. The amount of unbound E2 was assayed by absorptiometry based on competition with HRP-labeled E2 to anti-E2 antibody as described in the text.

4. Discussion

Our previous study demonstrated that TAM could be metabolized by quaternary ammonium-linked glucuronidation [13]. The present study provides evidence that *trans*-4-HO-TAM, an active metabolite of TAM, also can be metabolized by *N*-linked glucuronidation in human liver microsomes in vitro. N^+-Glucuronides formed in the reaction mixture consisting of human liver microsomes and 4-HO-TAM isomers in the

presence of UDPGA were identified with the synthetic specimens by HPLC–ESI-TOF-MS. Substrate specificity of 12 isoforms of human hepatic and extrahepatic UGTs indicated that *N*-glucuronidation of 4-HO-TAM isomers in human liver microsomes was catalyzed only by UGT1A4. The similar K_m values for *N*-glucuronidation by human liver microsomes and by recombinant UGT1A4 also indicated that UGT1A4 was responsible for human hepatic microsomal *N*-glucuronidation of *trans*-4-HO-TAM.

At least 17 UGT mRNAs are known to exist in the human. These are divided into two families, UGT1 and UGT2, consisting of 9 and 8 isoforms, respectively, on the basis of amino acid sequence identity. Among these isoforms, we used 8 isoforms of the UGT1A subfamily, UGT1A1 [24,25], UGT1A3 [26], UGT1A4 [24], UGT1A6 [27], UGT1A7 [28], UGT1A8 [29], UGT1A9 [30] and UGT1A10 [28], and 4 isoforms of the UGT2B subfamily, UGT2B4 [31–33], UGT2B7 [32,34–36], UGT2B15 [37,38], and UGT2B17 [39,40], which are currently commercially available. Both geometrical isomers of 4-HO-TAM were conjugated to form quaternary ammonium-linked glucuronides only by UGT1A4, although UGT1A4 had no activity for *O*-glucuronidation. Among isoforms having *O*-glucuronidating activity, UGT2B15 catalyzed most efficiently *O*-glucuronidation of 4-HO-TAM in a *cis*-selective manner as previously reported [10]. However, *O*-glucuronidating activity of human liver microsomes toward *cis*-4-HO-TAM was much higher than that by UGT2B15, which had the highest activity toward this substrate. The higher activity for *O*-glucuronidation toward *cis*-4-HO-TAM by human liver microsomes indicated that the baculovirus expression system, used for the expression of UGT2B15, produced a lesser amount of UGT2B15 protein in the insect cell microsomes than that existed in human liver microsomes. We have no evidence of a role in *N*-glucuronidation of 4-HO-TAM by human hepatic UGT2B10, UGT2B11, and UGT2B28, all of which were commercially unavailable and were not tested in this study.

Because of the high activity of *O*-glucuronidation toward *cis*-4-HO-TAM, only a small or trace amount of N^+-glucuronide was formed by human liver microsomes at 25 μM substrate concentration. Although a quantifiable amount of N^+-glucuronide was detected at the higher substrate concentration (100 μM), the reaction rate was approximately 1/50th of that of

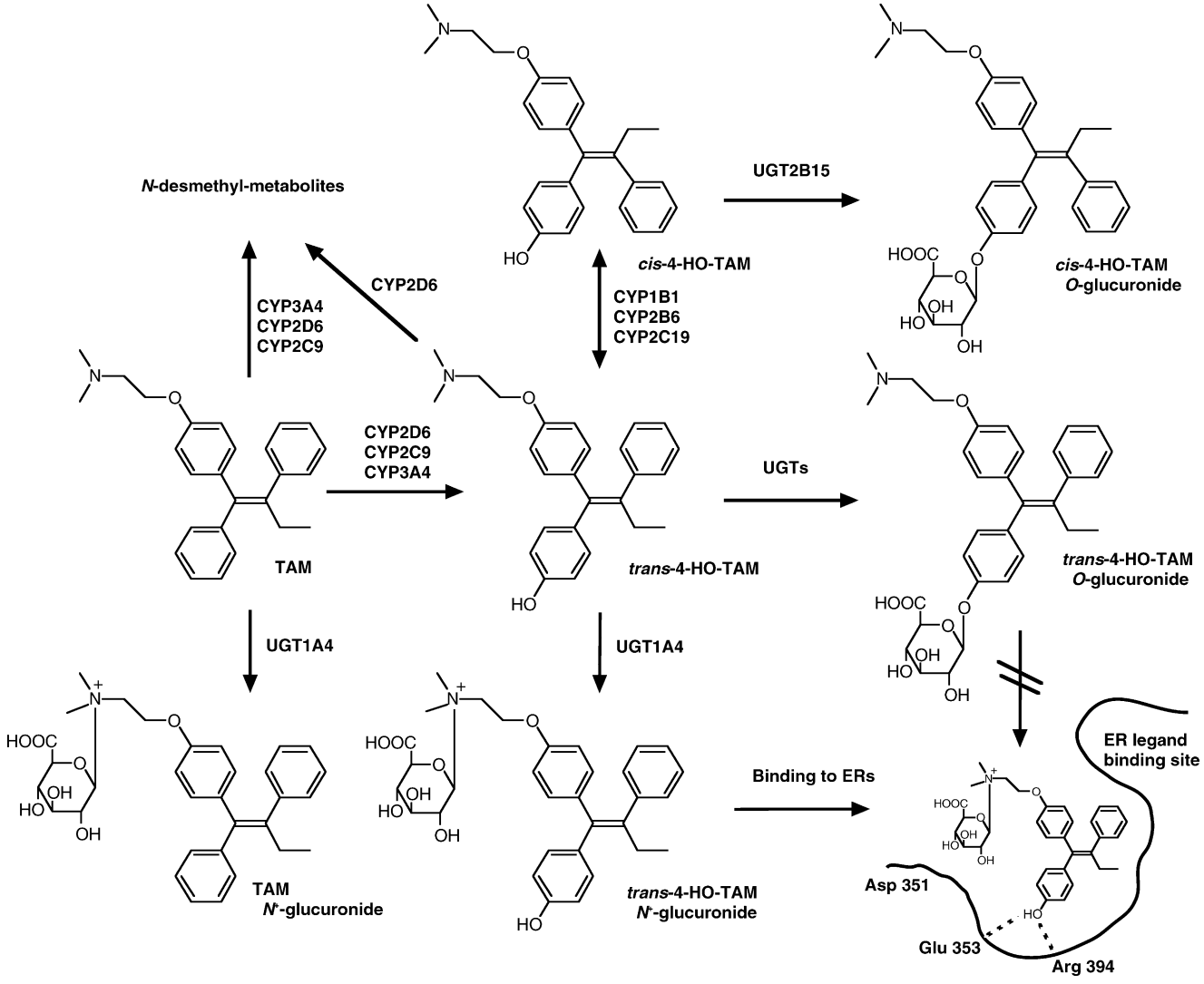

Fig. 5 – Proposed metabolic pathways of TAM and binding of 4-HO-TAM N^+-glucuronide to ER. Amino acids that interact with 4-HO-TAM have been identified based on the crystal structure of the 4-HO-TAM:ER complex [47].

O-glucuronidation, indicating that *N*-glucuronidation may not contribute to metabolism of the *cis*-isomer. In contrast, *trans*-4-HO-TAM was conjugated by *N*-linked glucuronidation at a rather higher rate than that by *O*-glucuronidation, suggesting that the active isomer could also be metabolized via *N*-glucuronidation (Fig. 5).

Liver microsomes from experimental animals such as rats, mice, monkeys, dogs, and guinea pigs failed to produce detectable amounts of 4-HO-TAM N^+-glucuronide under the same incubation conditions. Only rabbit microsomes had *N*-glucuronidating activity toward 4-HO-TAM isomers. *N*-glucuronidation of primary, secondary, and tertiary amines is known to be catalyzed mainly by human UGT isoforms, UGT1A3 and/or UGT1A4. *N*-Glucuronidation of antihistamic and antidepressant drugs, e.g., clozapine, chlorpromazine, loxapine, amitriptyline, imipramine, and (*R*)- and (*S*)-ketotifens were identified to be catalyzed by both isoforms UGT1A3 and UGT1A4 [41–43]. Other than human UGT1A3 and UGT1A4, only two rabbit UGT isoforms have been shown to catalyze glucuronidation of tertiary amines [44], whereas *N*-glucuronidation of primary amines occurs in many species [43]. Therefore, these rabbit isoforms may catalyze *N*-linked glucuronidation of 4-HO-TAM. Moreover, to investigate in vivo metabolism of TAM and its metabolites, the rabbit may be a candidate as an experimental animal. However, rabbit microsomes showed *trans*-selective *O*-glucuronidating activity toward 4-HO-TAM, resulting in an approximately 60-fold higher activity in *O*-glucuronidation of *trans*-4-HO-TAM than that of human microsomes, which indicated the existence of a large species difference between rabbits and humans. In the rat, the reason for lack of *N*-glucuronidating activity is suggested to result from the deficiency of an exon encoding the *N*-terminal part of the UGT isoform corresponding to human UGT1A4. The mechanism for the lack of N^+-glucuronidation in other species remains unclear. Therefore, especially, quaternary ammonium-linked glucuronidation is species-dependent and may be a factor in the species difference in the elimination pathway of many tertiary amines between humans and experimental animals.

Recent studies indicated that *N*-desmethyl-4-HO-TAM, one of the major TAM metabolites, also had similar affinity for ERs and may participate in the antiestrogenic activity of TAM due to its higher plasma concentration than that of 4-HO-TAM [11,45]. Therefore, we also investigated *N*-glucuronidation of *N*-desmethyl-TAM. However, neither human liver microsomes nor recombinant UGT isoforms were shown to have *N*-glucuronidating activity toward this substrate (data not shown). Although *N*-glucuronidation of secondary amines was reported in glucuronidation of diphenylamine and desmethylclozapine [41], *N*-demethylation of tertiary amines often lost their capability to receive *N*-glucuronidation [43]. Therefore, *N*-glucuronidation may not be involved in the metabolism of *N*-desmethyl-derivatives of TAM.

Trans-4-HO-TAM N^+-glucuronide was found to have affinities very similar or slightly stronger than *trans*-4-HO-TAM itself for human ERα and ERβ as a competitor to endogenous E2 (Fig. 4), suggesting that *N*-glucuronidation of *trans*-4-HO-TAM may not only be used for a deactivation reaction, but also could modify its pharmacological activity (Fig. 5). In contrast, *O*-glucuronidation of *trans*-4-HO-TAM greatly reduced its relative binding affinity for human ERs as reported for MCF-7 cytosolic ERs [14], which indicated that *O*-glucuronidation of *trans*-4-HO-TAM represented solely a deactivation pathway. In addition, we did not determine binding affinity of *cis*-4-HO-TAM N^+-glucuronide because *N*-glucuronidation may not contribute to the metabolism of the *cis*-isomer as discussed above, although the N^+-glucuronide may have binding activity for ERs.

In view of the structure-binding activity relationship, it was reported that antiestrogens, such as *trans*-4-HO-TAM and raloxifene, require an alkylaminoethoxy side chain to block estrogen action [46]. The presence of a *N*,*N*-dimethyl-alkyl side chain of TAM is not essential for binding to ERs as shown by the finding that *N*-demethylation or cleavage of the side chain from TAM reduces but not abolishes ER binding affinity [6]. The crystal structures of *trans*-4-HO-TAM:ER and raloxifene:ER complexes have been solved and showed that amino acids Glu353 and Arg394 interacted with the 4-hydroxyl group of 4-HO-TAM and that the antiestrogenic side chain interacts with Asp351 in the ER [47]. The structure of the ER-ligand complex indicated that binding of a bulky glucuronic acid moiety might sterically hinder the binding of 4-HO-TAM *O*-glucuronide to ER (Fig. 5).

Mechanistic studies of antiestrogen action using ER mutants indicated that the amino acid residue Asp351 in the ERα was critical for interactions with the antiestrogenic side chain of the antiestrogens [47]. Therefore, it is possible that the *N*-glucuronidation of *trans*-4-HO-TAM might modulate the antiestrogenic activity of this active metabolite of TAM (Fig. 5). Further study will be required to determine whether *trans*-4-HO-TAM N^+-glucuronide acts as an antagonist or agonist for ER transcriptional activity. Furthermore, it should be determined whether the N^+-glucuronide has biological significance in TAM therapy.

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