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Reverse geometrical selectivity in glucuronidation and sulfation of *cis*- and *trans*-4-hydroxytamoxifens by human liver UDP-glucuronosyltransferases and sulfotransferases

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Abstract

The phenolic active metabolites, *cis*-4-hydroxytamoxifen (*cis*-HO-TAM) and *trans*-4-hydroxytamoxifen (*trans*-HO-TAM), of the antibreast cancer drug, *trans*-tamoxifen (TAM), were geometrically selectively glucuronidated in the manner of *cis* ≫ *trans* by microsomes and sulfated in the manner of *trans* ≫ *cis* by cytosol from the liver of 10 human subjects (7 females and 3 males). There was a large individual difference in the microsomal glucuronidation of *cis*-HO-TAM, which correlated well with glucuronidation of 4-hydroxybiphenyl by human liver microsomes. However, there was only a slight correlation between the glucuronidation of *cis*-HO-TAM and *trans*-HO-TAM or 4-nitrophenol (NP). A small individual difference was observed for the human liver cytosolic sulfation of *trans*-HO-TAM, which correlated well with the sulfation of NP. Recombinant human UDP-glucuronosyltransferase (UGT)2B15 catalyzed the *cis*-selective glucuronidation of geometrical isomers of HO-TAM. UGTs1A1, 1A4, 1A9 and 2B7 had weak activity toward HO-TAMs with a much smaller *cis*-selectivity than did UGT2B15. UGTs1A3 and 1A6 had no detectable activity toward these substrates. Among the four known major sulfotransferases (SULTs) occurring in the human liver, SULT1A1 was strongly suggested to play the most important role in the hepatic cytosolic *trans*-selective sulfation of HO-TAM isomers. A good correlation was observed between the hepatic cytosolic sulfation of *trans*-HO-TAM and NP, a standard substrate for SULT1A1. SULT1E1 had slight activity toward the HO-TAMs. SULTs1A3 and 2A1 had no detectable activity toward HO-TAMs.

Keywords: 4-Hydroxytamoxifen; UDP-glucuronosyltransferase; Sulfotransferase; Human liver microsomes; Human liver cytosol; Geometrical selectivity

1. Introduction

Trans-4-hydroxytamoxifen (1-[4-(2-dimethylaminoethoxy)phenyl]-1-(4-hydroxyphenyl)-2-phenylbut-1(*Z*)-ene) is recognized as an active metabolite of the antibreast cancer drug, *trans*-tamoxifen (TAM, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene) (Fig. 1), which is most widely used for chemotherapy and chemoprevention of breast cancer [1]. Although *trans*-HO-TAM was identified as a major serum component in humans with a circulating level less than 10% of that attained by the parent drug. Fabian *et al.* [2] suggested that the low serum levels of *trans*-HO-TAM may be sufficient, in view of its activity, to contribute significantly to the net effect of TAM. It was reported that the estrogen antagonist, *trans*-HO-TAM, was approximately 100-fold more interactive with human estrogen receptors than TAM [3,4]. Other major TAM metabolites, *N*-desmethyl-TAM, *N*-desdimethyl-TAM, and a primary alcohol named metabolite Y, showed

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Abbreviations: BSA, bovine serum albumin; DHEA, dehydroepiandrosterone; DMSO, dimethyl sulfoxide; E_2, β-estradiol; GA, glucuronic acid; HO-BP, 4-hydroxybiphenyl; HO-TAM, 4-hydroxytamoxifen; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; NP, 4-nitrophenol; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; SULT, sulfotransferase; TAM, *trans*-tamoxifen; TLC, thin-layer chromatography; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

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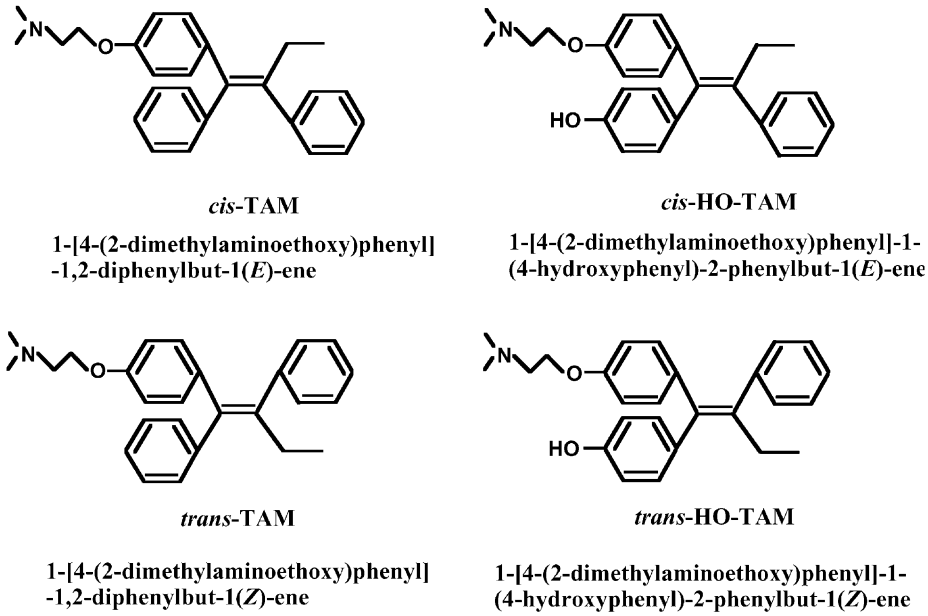

Fig. 1. Structures and IUPAC nomenclature of *cis*- and *trans*-TAMs and HO-TAMs.

lower *in vitro* affinity for the human estrogen receptors than that of TAM. Aromatic hydroxylation of TAM to *trans*-HO-TAM is mediated by human cytochrome P-450s (CYPs) 2D6, 2C9, and 3A4 [5,6]. The geometrical isomer, *cis*-HO-TAM, of *trans*-HO-TAM, was demonstrated to be an estrogen agonist and to be a metabolite of *trans*-HO-TAM by human liver microsomes in the presence of NADPH [7]. The microsomal geometrical inversion of *trans*-HO-TAM to the *cis*-isomer appears to be mediated by CYPs as it was inhibited by SKF525-A [7]. It is of interest that *cis*-HO-TAM existed at a higher concentration than *trans*-HO-TAM in the breast carcinoma of TAM-administered female patients who had become resistant to TAM during the course of antibreast cancer chemotherapy [8,9].

To our knowledge, no information is available on the hepatic glucuronidation and sulfation of the geometrically isomeric phenolic substrates, *cis*- and *trans*-HO-TAMs, in the human, whereas biliary and urinary excretions of a *trans*-HO-TAM glucuronide have been demonstrated in both female humans and rats administered TAM [10,11].

Falany [12] showed *trans*-HO-TAM to be sulfated by the bacterially expressed human phenol and estrogen SULTs, SULTs1A1 and 1E1, respectively. However, no evidence has been provided for the sulfation of *cis*-HO-TAM by these human SULTs and for the *in vivo* formation of sulfates of *cis*- and *trans*-HO-TAMs in the human and in animals.

In the present paper, we provide the first evidence for the geometrically selective glucuronidation of *cis*-HO-TAM and sulfation of *trans*-HO-TAM by human liver microsomes and cytosol, respectively. Evidence will be also provided that the geometrically selective glucuronidation and sulfation of HO-TAM isomers are mainly catalyzed by human UGT2B15 and SULT1A1, respectively.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), β-glucuronidase (from *Escherichia coli*, 100 units/mL), 4-hydroxybiphenyl (HO-BP), HO-TAM, NP, and UDP-α-D-glucuronic acid (UDPGA) were purchased from Sigma Chemical Co. Unlabeled 3′-phosphoadenosine 5′-phosphosulfate (PAPS) was synthesized as reported previously [13]. [2,4,6,7-^{3}H]β-estradiol (2.96 TBq/mmol) and [^{35}S]PAPS (50.5 GBq/mmol) were purchased from NEN Life Science Products, Inc. Radioactive PAPS was purified by chromatography on a DEAE–cellulose column prior to use by a previously reported method [14]. [^{14}C-GA]UDPGA (9.91 GBq/mmol) was purchased from ICN Pharmaceuticals. *E. coli* BL21(DE3)pLysS strain, His. Bind Resin, and pET-14b expression vector were purchased from Novagen. Microsomes prepared from insect cells individually expressing recombinant human UGTs1A1, 1A3, 1A6, and 2B7 in Sf9 cells were purchased from PanVera Co, and those from insect BTI-TN-5B1-4 cells expressing human recombinant UGTs1A4, 1A9, and 2B15 were from Gentest Co. Glucuronidating activities of the UGTs were measured according to the manufacturer's instructions using the indicated substrates, octyl gallate (UGTs1A1 and 1A3), trifluoroperazine (UGT1A4), 1-naphthol (UGT1A6), hyodeoxycholic acid (UGT2B7) and 7-hydroxy-4-trifluoromethylcoumarin (UGTs1A9 and 2B15) and by our systems using usual substrates (described here). HO-TAM obtained from Sigma Chemical Co 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*-HO-TAMs with purity more than 98 and 99%,

respectively. The *cis*- and *trans*-HO-TAMs were eluted at retention times of 7.9 and 9.0 min from the HPLC column, and identified by ^{1}H-NMR spectroscopy with an NMR spectrometer model MERCURY 300 (Varian Inc.) as reported previously [17] and by mass spectrometry with a mass spectrometer model TSQ 700 (ThermoQuest). Other reagents used were of analytical grade.

2.2. Human liver samples

Ten cancer patients (three males and seven females; mean age, 59.7; range 29–76), hospitalized at the National Cancer Center Hospital, Tokyo, Japan, were entered into the present study. 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 underwent 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 the removed tissue. All of the fresh samples were rapidly frozen in liquid nitrogen and stored at −80° before use.

2.3. Preparation of human liver microsomes and cytosols

Approximately 1 g of human liver was homogenized in 3 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°, and the supernatant was collected. The supernatant was centrifuged at 105,000 *g* for 60 min at 4°, and the resulting supernatant fraction was used as the cytosolic fraction. The pellet was resuspended in 1 mL of the same buffer and used as the microsomal fraction. The cytosolic and microsomal fractions were stored at −80° before use. Protein concentrations of these fractions were measured by the method of Bradford [19] using BSA as a standard.

2.4. Preparation of human recombinant SULTs

The entire coding region of human SULTs1A1 (Genbank accession no. X78283), 1A3 (Genbank accession no. L19956), 1E1 (Genbank accession no. L25275), and 2A1 (Genbank accession no. U08025) were amplified by PCR from a human liver cDNA library [20]. The amplified SULT1A1 cDNA sequence in the present study was identical with *SULT1A1∗1* reported by Ozawa *et al.* [21], which was the most abundant of three reported *SULT1A1* alleles in the human population [22]. The cDNAs were subcloned into the *Nde* I/*Xho* I sites (SULTs1A1, 1A3, and 1E1) and *Nde* I site (SULT2A1) of the pET-14b expression vector. The resulting expression plasmid was used for transformation of *E. coli* BL21(DE3)pLysS. Expression of His-tagged SULTs and purification of the enzymes from the bacterial lysate were performed according to a previously described method with a modification [23]. In brief, the lysate was loaded on a His Bind Resin column (1 cm × 5 cm) pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 5 mM imidazole, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.025% (w/v) Tween 20, and 10% (w/v) glycerol (buffer I). The recombinant protein was eluted with a step-wise gradient of 15 mL each of 100, 200, 400, and 1000 mM imidazole in buffer I, after the column was washed with 60 mL of buffer I. The purified enzyme was desalted on a PD-10 column (16 mm × 50 mm, Amersham Pharmacia Biotech) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.8) containing 8 mM dithiothreitol, 0.025% (w/v) Tween 20, and 10% (w/v) glycerol. Protein concentration was measured by the method of Bradford [19] using BSA as a standard, and the purified SULTs were stored at −80° after the addition of BSA (1%, w/v) as an SULT stabilizer [23].

2.5. Enzyme assay

Glucuronidating activity of human liver microsomes and insect cell microsomes expressing UGT isoforms toward HO-BP, *cis*- and *trans*-HO-TAMs, and NP were determined as described previously [24] with the following modifications. For all of the assays, human liver microsomes were activated by a 30-min incubation in an ice bath with Triton X-100 at a detergent-to-protein weight ratio of 0.4. This step was omitted for microsomes from insect cells expressing UGT isoforms, because the treatment of insect microsomes with Triton X-100 rather inhibited their enzyme activity. A reaction mixture containing the substrate dissolved in dimethyl sulfoxide (DMSO) (2.5 μL), 50 mM Tris–HCl buffer (pH 7.5), 10 mM $MgCl_2$, and microsomal protein (2–40 μg) at a final volume of 50 μL was incubated for 30 min at 37°. The reaction was started by the addition of 2 mM [^{14}C-GA]UDPGA (specific activity: 146 MBq/mmol) and terminated by the addition of 50 μL of methanol. After centrifugation at 12,000 *g* for 5 min, 40 μL of the supernatant was applied onto a silica gel 60 F254 thin layer plate (Merck) having a preabsorbent sample-spotting area, and developed with *n*-butanol/acetone/glacial acetic acid/30% (w/v) ammonia/water (70:50:18:1.5:60, v/v). The radioactivity of the glucuronide was measured by radio luminography with a BAS2000 bioimaging analyzer (Fuji Photo Film). The DMSO concentration (5%, v/v) did not affect the UGT activities of human and insect microsomes toward all substrates used in the present study. Data were expressed as mean values obtained from at least three replicated incubations. The sulfating activity of human liver cytosol and purified recombinant human SULTs toward dehydroepiandrosterone (DHEA), dopamine, *cis*- and *trans*-HO-TAMs, and NP were determined as described previously [14] with the following modifications. The reaction mixture contained [^{35}S]PAPS (10 μM, 3.4 MBq/mmol), 7 mM $MgCl_2$, 8 mM dithiothreitol, 0.0625% (w/v) BSA, and hepatic cytosol (10–20 μg protein) or a purified enzyme

(5–300 ng) in a final volume of 47.5 μL of 50 mM sodium phosphate buffer (pH 6.8 for SULTs1A1 and 1A3) or 50 mM Tris–HCl buffer (pH 7.4 for cytosol, SULTs1E1 and 2A1). Following incubation of the mixture without the substrate for 2 min at 37°, the reaction was started by the addition of the substrate dissolved in DMSO (2.5 μL). For determination of kinetic parameters for the sulfation of *cis*- and *trans*-HO-TAMs, substrate concentrations ranging 5–200 μM were used. After a 10-min incubation at 37°, the reaction was terminated by placement of the reaction vessel in an ice bath, and 5 μL of the reaction mixture was applied onto a polyethyleneimine cellulose thin layer-plate (Marcherey–Nagel GmbH & Co) and developed with *t*-butanol:ethyl acetate:water (8:6:4, v/v). The radioactivity of the sulfate formed was determined by radio luminography as described above. Sulfating activity of SULT1E1 toward β-estradiol (E_2) was determined as reported previously [25]. For calculation of k_{cat} values, molecular weights of the His-tagged SULT proteins used were 36,372 for SULT1A1, 36,358 for SULT1A3, 37,288 for SULT1E1, and 35,927 for SULT2A1. The DMSO concentration (5%, v/v) did not affect the SULT activities of human cytosol and recombinant SULTs toward substrates used in the present study. Data were expressed as mean values of at least three replicated incubations.

2.6. Identification of glucuronides formed from geometrical isomers of HO-TAM

For identification of radioactive glucuronides formed from geometrical isomers of HO-TAM by human liver microsomes in the presence of [^{14}C-GA]UDPGA, the final volume of the incubation mixture was increased up to 1 mL without changing the concentrations of the constituents. After incubation for 1 hr at 37°, the reaction was terminated by the addition of 1 mL of methanol, and the mixture was centrifuged at 12,000 *g* for 5 min. The resulting supernatant was dried by vacuum centrifugation at 40°, and the resultant residue was dissolved in water and loaded onto a Sep-pak cartridge (Waters Co). The cartridge was washed with 5 mL of water and then eluted with 2 mL of methanol. The methanolic effluent from the cartridge was concentrated to dryness at 40° in a gentle stream of nitrogen and redissolved in 200 μL of an HPLC mobile phase consisting of 60% (v/v) methanol in 0.25 M ammonium acetate buffer (pH 5.0). The solution was loaded onto an Inertsil ODS-3 column (4.6 mm × 150 mm, 5 μm; GL Science, Tokyo, Japan) and eluted at a flow rate of 1 mL/min with a 60–80% (v/v) linear gradient of methanol (0.67% min^{-1}) in 0.25 M ammonium acetate buffer (pH 5.0). The elution of the unreacted substrate and radioactive glucuronide was monitored by absorbance at 254 nm. Radioactivity of the chromatographic fractions was measured with a bioimaging plate/BAS2000 bioimazing analyzer as reported previously [26]. Chromatographic fractions containing the radioactive glucuronide were pooled, and the solvent was evaporated to dryness *in vacuo* at 40°. 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 hr at 37°. The incubation mixture was filtered through a disc filter (Kanto Co) and subjected to HPLC performed under the same conditions as described above. Mass spectrometric analysis of the aglycons from the radioactive glucuronides was performed with a mass spectrometer model TSQ 700 (ThermoQuest).

3. Results

3.1. Cis-selective glucuronidation of cis- and trans-HO-TAMs by human liver microsomes

Cis- and *trans*-HO-TAMs were both transformed to radioactive glucuronides in the presence of [^{14}C-GA]UDPGA by human liver microsomes (Fig. 2). The radioactive glucuronides from *cis*- and *trans*-HO-TAMs were eluted as UV-absorbing peaks at retention times of 7.5 and 6.5 min, respectively, from a reverse phase HPLC column (Fig. 2A and B). Treatment with bacterial β-glucuronidase of the radioactive glucuronides eluted from the HPLC column afforded non-radioactive UV-absorbing products, which were identified as *cis*- and *trans*-HO-TAMs by HPLC (Fig. 2C and D) with the respective authentic specimens followed by MS (data not shown). No appreciable geometrical inversion between *cis*- and *trans*-HO-TAMs took place under the above incubation and HPLC conditions.

A TLC-radioluminographic study indicated that the hepatic microsomal glucuronidation of HO-TAM isomers proceeded in the manner of *cis* ⩾ *trans* in all seven females (F1–F7) and three males (M1–M3) examined (Table 1). Ratios of *cis*- to *trans*-glucuronides formed from HO-TAM isomers was 113–370:1 at a substrate concentration of 5 μM and 52–215:1 at a substrate concentration of 25 μM in these human subjects. The rate of hepatic microsomal *cis*-HO-TAM glucuronidation varied significantly in the 10 subjects, and the conjugation proceeded 10-fold faster at substrate concentrations of 5 and 25 μM in M3 with the highest hepatic UGT activity than in F1 with the smallest activity of the 10 subjects. All the three males showed higher glucuronidating activity toward *cis*-HO-TAM than all the seven females, whereas no significant difference was observed in *trans*-HO-TAM glucuronidation between the males and females. *Cis*-HO-TAM was a good substrate comparable to NP and HO-BP used as standard substrates for UGTs (Table 1).

In all the subjects examined, there was a very good correlation ($r^2 = 0.97$) between the rates of hepatic microsomal glucuronidation of 25 μM *cis*-HO-TAM and HO-BP (Fig. 3A) based on the data listed in Table 1. However, no correlation ($r^2 = 0.09$) was observed between the glucuronidation of *cis*-HO-TAM and NP (Fig. 3B). A moderate

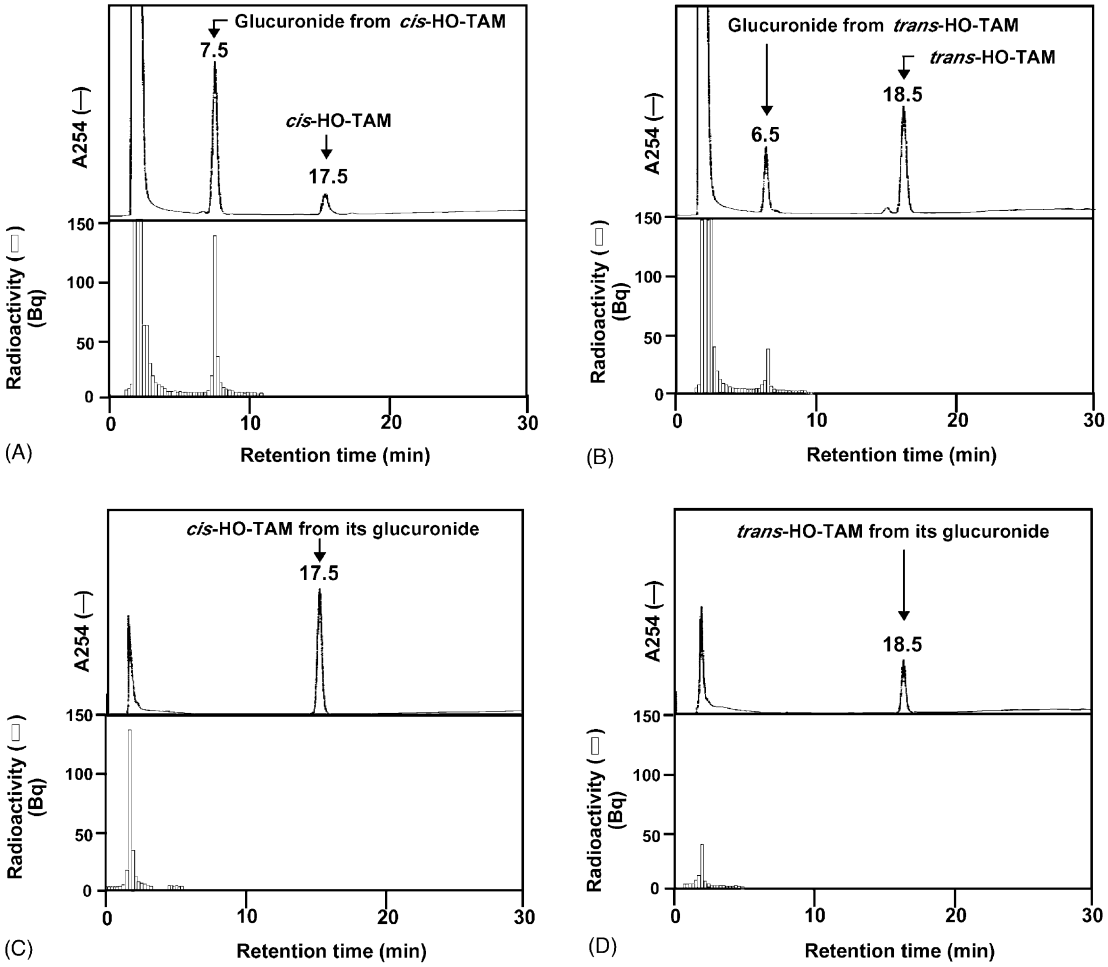

Fig. 2. HPLC of radioactive glucuronides formed from *cis*- and *trans*-HO-TAMs by human liver microsomes in the presence of [^{14}C-GA]UDPGA and of aglycons released from the glucuronides by treatment with β-glucuronidase. Chromatograms were monitored by absorbance at 254 nm (upper) and by liquid scintillation counting of radioactivity in the column effluent collected at 1-min intervals (lower). Panels (A) and (B) represent UV-absorbing and radioactive glucuronides formed from *cis*- and *trans*-HO-TAMs, respectively, by incubation with human liver microsomes (from F4 in Table 1) in the presence of [^{14}C-GA]UDPGA. Panels (C) and (D) represent aglycons released by β-glucuronidase from the chromatographically isolated radioactive glucuronides of *cis*- and *trans*-HO-TAMs, respectively. Details are described in Section 2. Numerals in the chromatograms represent retention times. HPLC for panels (A–D) was performed on an Inertsil ODS-3 column eluted with 0.25 M ammonium acetate buffer (pH 5.0) and increasing amounts of methanol (0.67% (v/v)/min) in a linear gradient. The radioactivity eluted at void volume was attributable to unreacted [^{14}C-GA]UDPGA (panels A and B) and [^{14}C]GA (panels C and D).

correlation ($r^2 = 0.46$) between the glucuronidation of *trans*-HO-TAM and HO-BP was observed (Fig. 3C), but no correlation ($r^2 = 0.01$) was observed between *trans*-HO-TAM and NP (Fig. 3D). There was a moderate correlation ($r^2 = 0.56$) between the glucuronidation of *cis*- and *trans*-HO-TAMs.

At substrate concentrations higher than 25 μM, hepatic *cis*-HO-TAM glucuronidation was strongly inhibited by the substrate, although a weak substrate inhibition was observed for *trans*-HO-TAM glucuronidation at higher than 100 μM (Fig. 4A–C). Therefore, apparent K_m values for the glucuronidation of *cis*- and *trans*-HO-TAMs were extrapolated from Michaelis–Menten plots at concentrations ranging 1.25–25 μM and 1.25–100 μM for *cis*- and *trans*-HO-TAMs, respectively. Observed apparent K_m values for the microsomal *cis*- and *trans*-HO-TAMs glucuronidations were 9.2 and 50.0 μM in F4, 6.5 and 27.9 μM in F7, and 7.2 and 38.3 μM in M3, respectively.

3.2. *Trans*-selective sulfation of *cis*- and *trans*-HO-TAMs by human liver cytosol

The TLC-radioluminographic study indicated that in the presence of [^{35}S]PAPS, the geometrical isomers of HO-TAM were converted to radioactive sulfates in the manner of *trans* ⩾ *cis* by human liver cytosols (Fig. 4D–F and Table 2). The *trans* to *cis* ratios in the sulfation of HO-TAM isomers were 15–27:1 and 23–48:1 at substrate concentrations of 5 and 25 μM, respectively (Table 2). In contrast to microsomal *cis*-HO-TAM glucuronidation, no appreciable substrate inhibition was observed for the cytosolic sulfation of *trans*- and *cis*-HO-TAMs (Fig. 4D–F). In the same seven females and three males who were examined for microsomal glucuronidation, cytosolic sulfation of *trans*-HO-TAM proceeded with a much smaller individual difference than did the glucuronidation of *cis*-HO-TAM (Tables 1 and 2). Hepatic cytosolic *trans*-HO-TAM-sulfating

Table 1
Cis-selective glucuronidation of *cis*- and *trans*-HO-TAMs by human liver microsomes

Donor	Age	Specific activity (nmol/min/mg protein)							
		Cis-HO-TAM[a]		*Trans*-HO-TAM		*Cis/trans*		NP (25 μM)	HO-BP (25 μM)
		5 μM	25 μM	5 μM	25 μM	5 μM	25 μM		
F1	76	1.13	2.17	0.01	0.04	113	52	30.60	9.02
F2	47	1.32	3.12	0.01	0.02	265	156	16.15	9.54
F3	71	3.31	6.64	0.02	0.10	144	66	20.60	13.29
F4	46	3.46	6.80	0.01	0.06	314	117	13.50	11.46
F5	29	3.04	7.23	0.02	0.10	145	73	17.66	12.80
F6	50	4.30	8.89	0.02	0.08	253	117	25.67	13.92
F7	52	4.46	9.14	0.02	0.08	186	117	19.43	16.24
M1	54	6.50	14.52	0.03	0.15	210	96	20.89	19.69
M2	56	8.38	16.92	0.03	0.13	254	126	22.10	22.91
M3	67	10.74	20.60	0.03	0.10	370	215	29.31	28.86

An amount of 5 and 25 μM *cis*- and *trans*-HO-TAMs were incubated for 30 min with human liver microsomes (40–800 μg protein/mL) from seven females (F1–F7) and three males (M1–M3) in the presence of 2 mM [^{14}C-GA]UDPGA (146 mBq/mmol) in a final volume of 50 μL of 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.4% (w/v) Triton X-100 for activating UGTs and 5% (w/v) DMSO for dissolving the substrate. An amount of 25 μM NP and HO-BP were also used as standard substrates for determining microsomal UGT activity under the same incubation conditions as mentioned above. Radioactive glucuronides formed were separated from unreacted radioactive UDPGA on a silicagel plate by TLC and determined by radioluminography. Details are described in Section 2. Data are expressed as arithmetic mean values of at least three replicated incubations. Standard deviation of specific activities was less than 0.2 and 0.35 for 5 and 25 μM *cis*-HO-TAM, 0.004 and 0.02 for 5 and 25 μM *trans*-HO-TAM, 2.5 for NP, and 2.7 for HO-BP, respectively.

[a] Microsomal glucuronidation of *cis*-HO-TAM was strongly inhibited by the substrate at concentrations higher than 25 μM as shown in Fig. 4.

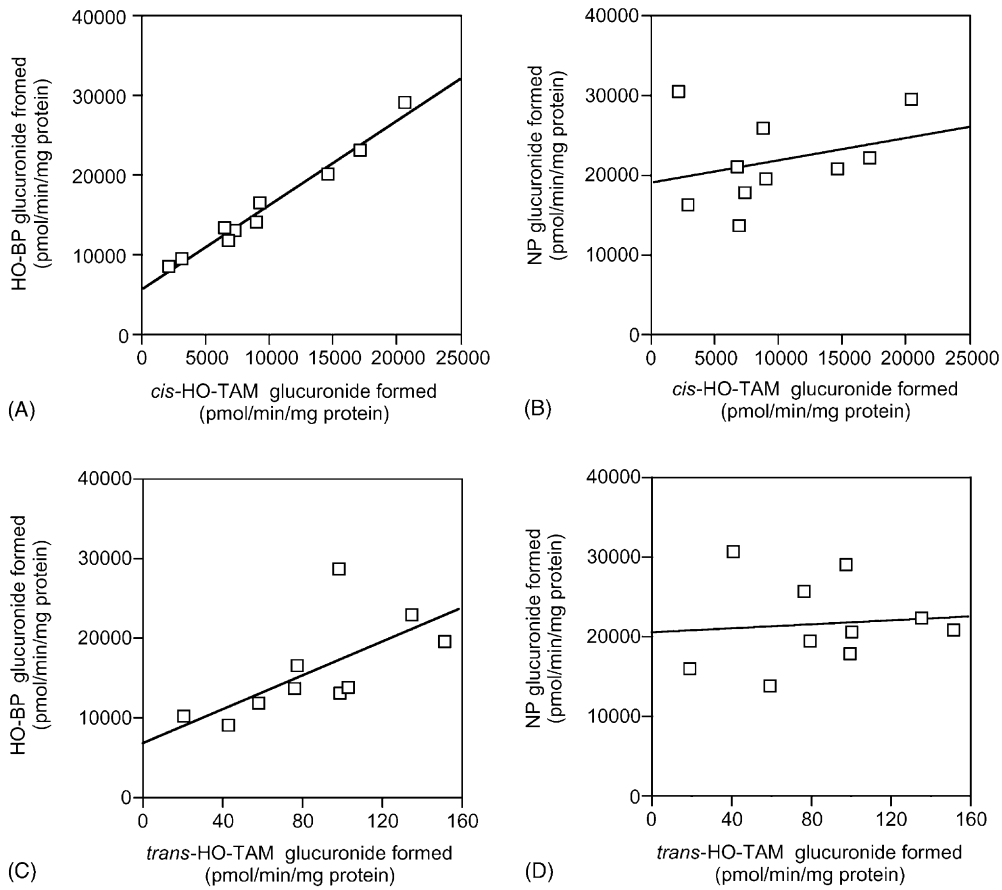

Fig. 3. Correlation between glucuronidation of *cis*- or *trans*-HO-TAM and NP or HO-BP by liver microsomes from 10 human subjects, F1–F7 and M1–M3. Data listed in Table 1 were used. The substrate concentration of HO-TAMs, HO-BP, and NP was 25 μM. Correlation efficiencies were as follows: panel (A), *cis*-HO-TAM- and HO-BP-glucuronidating activities ($r^2 = 0.97$); panel (B), *cis*-HO-TAM- and NP-glucuronidating activities ($r^2 = 0.09$); panel (C), *trans*-HO-TAM- and HO-BP-glucuronidating activities ($r^2 = 0.46$); and panel (D), *trans*-HO-TAM- and NP-glucuronidating activities ($r^2 = 0.01$).

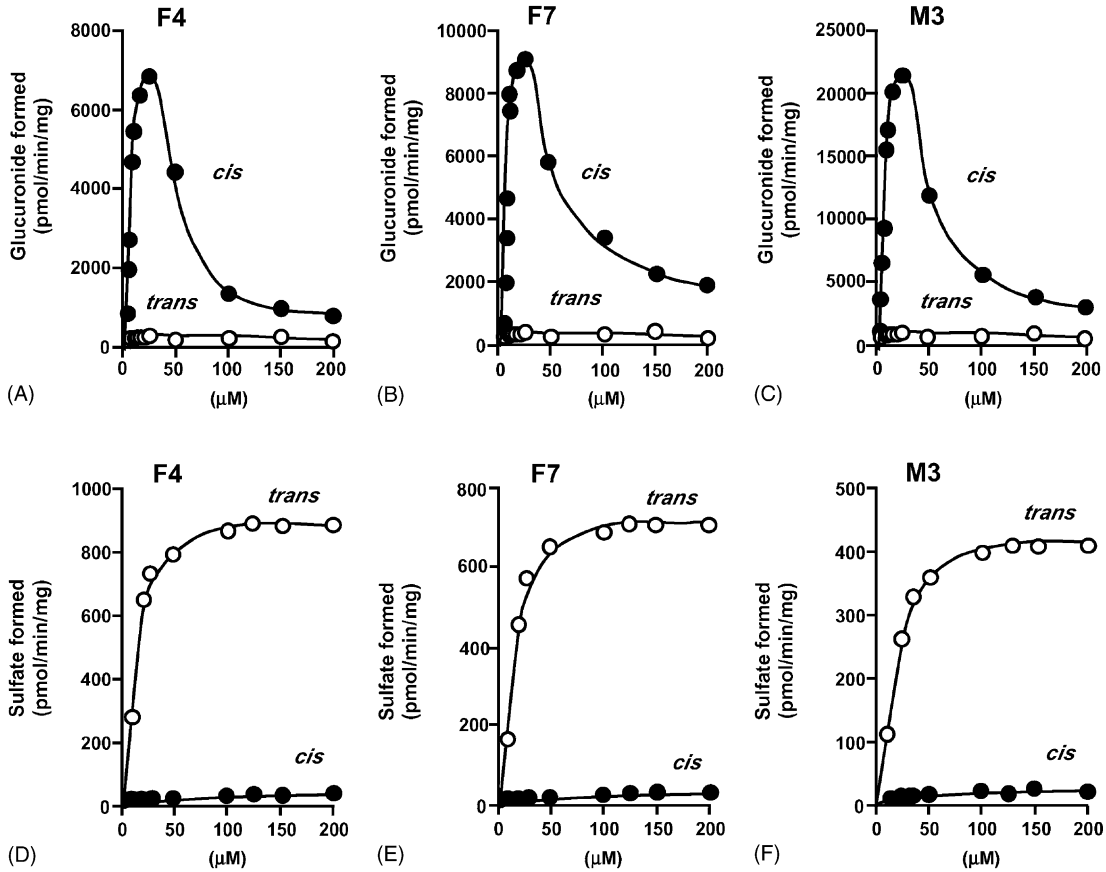

Fig. 4. Glucuronidation and sulfation of various concentrations of *cis*- and *trans*-HO-TAMs by human liver microsomes and cytosols. Panels (A–C), liver microsomes from two females (F4 and F7) and one male (M3) listed in Table 1 were incubated with various concentrations of *cis*-HO-TAM (●) and *trans*-HO-TAM (○) at 37° for 30 min in the presence of 2 mM [^{14}C-GA]UDPGA. Microsomal enzyme activities toward the geometrical isomers of HO-TAMs were determined as described in Table 1. Panels (D–F), liver cytosols from the donors noted above were incubated with various concentrations of *cis*- and *trans*-HO-TAMs at 37° for 10 min in the presence of 10 μM [^{35}S]PAPS. Cytosolic enzyme activities toward the geometrical isomers of HO-TAMs were determined as described in Section 2. Data are expressed as arithmetic mean values of at least three replicated incubations.

Table 2
Trans-selective sulfation of *cis*- and *trans*-HO-TAMs by human liver cytosol

Donor	Specific activity (pmol/min/mg protein)							
	Cis-HO-TAM		*Trans*-HO-TAM		*Trans/cis*		NP[a] (5 μM)	E_2[a] (20 nM)
	5 μM	25 μM	5 μM	25 μM	5 μM	25 μM		
F1	13.1	11.6	196	434	15.0	37.4	659	4.0
F2	6.7	12.7	145	332	21.6	23.2	465	3.4
F3	13.4	18.4	222	428	16.5	23.3	525	5.3
F4	13.7	24.5	285	624	20.8	25.5	820	2.0
F5	9.0	10.3	239	496	26.5	48.2	633	7.5
F6	11.6	13.9	151	371	13.0	26.7	529	5.4
F7	11.8	15.2	186	460	15.8	30.3	678	4.6
M1	8.2	13.4	158	382	19.3	28.5	514	5.3
M2	8.5	13.5	176	409	20.7	30.3	534	4.7
M3	6.7	11.6	108	272	16.2	23.4	467	3.0

An amount of 5 and 25 μM *cis*- and *trans*-HO-TAMs were incubated for 10 min in the presence of 10 μM [^{35}S]PAPS (14.8 mBq/mmol) with human liver cytosol (0.2–0.4 mg protein/mL) from seven females (F1–F7) and three males (M1–M3) in a final volume of 50 μL of 50 mM Tris–HCl buffer (pH 7.4) containing 7 mM $MgCl_2$, 8 mM dithiothreitol, 0.0625% (w/v) BSA, and 5% (w/v) DMSO for dissolving the substrate. NP (5 μM) and E_2 (20 nM) were also used as substrates under the incubation conditions as described in Section 2. Radioactive sulfates formed were separated from the unreacted radioactive PAPS on a PEI cellulose plate by TLC and determined by radioluminography. Details are described in Section 2. Data are expressed as arithmetic mean values of at least three replicated incubations. Standard deviation of specific activities was less than 2.4 and 2.6 for 5 and 25 μM *cis*-HO-TAM, 18 and 46 for 5 and 25 μM *trans*-OH-TAM, 24 for NP, and 0.4 for E_2, respectively.

[a] Sulfation was strongly inhibited by substrates at concentrations higher than 5 μM for NP and 20 nM for E_2.

activity was approximately only 2-fold higher in donor F4 than in donor M3, who had the highest and lowest enzyme activities, respectively, among the 10 subjects examined. Therefore, there was no correlation between hepatic microsomal *cis*-HO-TAM-glucuronidating and cytosolic *trans*-HO-TAM-sulfating activities ($r^2 = 0.18$) in these 10 subjects based on the data listed in Tables 1 and 2. Apparent K_m values extrapolated from Michaelis–Menten plots for the cytosolic *cis*- and *trans*-HO-TAM sulfations were 52.3 and 14.1 μM in F4, 36.0 and 21.1 μM in F7, and 48.4 and 21.5 μM in M3, respectively.

A good correlation ($r^2 = 0.85$) was observed between the hepatic cytosolic sulfation of *trans*-HO-TAM and NP, a typical substrate for SULT1A1, although there was a weak correlation ($r^2 = 0.35$) between the sulfation of *cis*-HO-TAM and NP (Fig. 5A and C) based on the data listed in Table 2. No correlation was observed between the sulfation of *trans*- or *cis*-HO-TAM and E_2 (Fig. 4B and D). A moderate correlation was also observed between the sulfation of *cis*- and *trans*-HO-TAMs ($r^2 = 0.45$).

3.3. Geometrical selectivity in glucuronidation of *cis*- and *trans*-HO-TAMs by recombinant human UGTs

Seven microsomal recombinant human UGTs1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15, that were separately expressed in insect cells were used to investigate the geometrical selectivity in glucuronidation of HO-TAMs. Of these recombinant human enzymes in insect cell microsomes, UGTs1A1, 1A4, 1A9, 2B7, and 2B15 had glucuronidating activity toward HO-TAMs, whereas UGTs1A3 and 1A6 had no detectable activity toward the geometrical isomers (Table 3). However, UGTs1A3 and 1A6 showed considerable activity toward NP and HO-BP under the same incubation conditions. UGT2B15 was 1.9-fold more and 0.4-fold less active toward *cis*-HO-TAM than toward NP and HO-BP, respectively. UGT1A4 was 2.4- and 2.9-fold more active toward *cis*-HO-TAM than toward NP and HO-BP, respectively. UGTs1A1 and 2B7 were much less active toward *cis*-HO-TAM than toward HO-BP. The *cis* to *trans* ratio was 12:1 for the glucuronidation of HO-TAMs

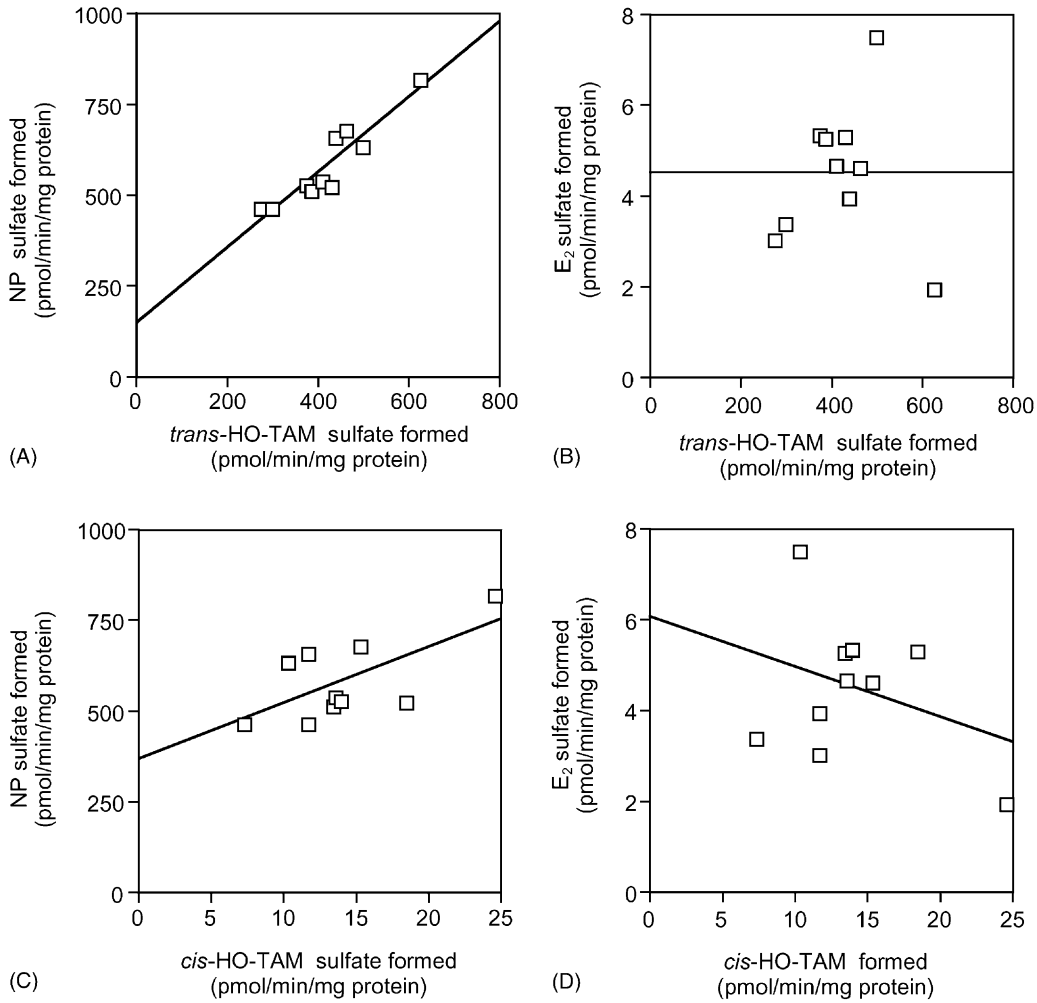

Fig. 5. Correlation between sulfation of *cis*- or *trans*-HO-TAM and NP or E_2 by liver cytosol from 10 human subjects, F1–F7 and M1–M3. Data listed in Table 2 were used. The substrate concentration of HO-TAMs was 25 μM. Correlation efficiencies were as follows: panel (A), *trans*-HO-TAM- and NP-sulfating activities ($r^2 = 0.85$); panel (B), *trans*-HO-TAM- and E_2-sulfating activities ($r^2 = 0.00$); panel (C), *cis*-HO-TAM- and NP-sulfating activities ($r^2 = 0.35$); and panel (D), *cis*-HO-TAM- and E_2-sulfating activities ($r^2 = 0.24$).

Table 3
Glucuronidation of *cis*- and *trans*-HO-TAMs by recombinant human UGTs expressed in insect cells

Human UGT	Specific activity (pmol/min/mg protein)				
	Cis-HO-TAM	*Trans*-HO-TAM	*Cis/trans*	NP	HO-BP
1A1	20.2	13.3	1.5	49.4	221.9
1A3	N.D.[a]	N.D.		10.0	9.5
1A4	48.3	56.8	0.9	20.0	16.4
1A6	N.D.	N.D.		675.5	17.1
1A9	31.8	9.7	3.3	2039.7	653.1
2B7	9.7	7.2	1.3	62.2	134.0
2B15	382.8	31.2	12.3	200.5	874.6

An amount of 25 μM *cis*- and *trans*-HO-TAMs, NP, and HO-BP were incubated in the presence of 2 mM [^{14}C-GA]UDPGA with microsomes (40–800 μg protein/mL) from insect cells expressing human UGTs, and radioactive glucuronides formed were separated and determined under the same conditions as described in Table 1. Data are expressed as arithmetic mean values of at least three replicated incubations.

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

at a substrate concentration of 25 μM by UGT2B15. The ratio was much smaller for UGTs1A1 (1.5:1), 1A4 (0.9:1), 1A9 (3.3:1) and 2B7 (1.3:1) than for UGT2B15.

Glucuronidation of *cis*-HO-TAM by UGT2B15 was remarkably inhibited with the substrate at concentrations higher than 25 μM (Fig. 6A) as observed in its glucuronidation by human liver microsomes (Fig. 4A–C). The *trans*-HO-TAM glucuronidation by UGT2B15 was also inhibited by the substrate at concentrations higher than 100 μM. Therefore, apparent K_m values, 7 and 18 μM, for the glucuronidation of *cis*- and *trans*-HO-TAMs by UGT2B15 were extrapolated from Michaelis–Menten plots at concentrations ranging 1.25–12.5 μM and 1.25–50 μM for the *cis*- and *trans*-isomers, respectively (Fig. 6B). The substrate inhibition was also observed in glucuronidation of HO-TAMs by UGTs1A4 and 1A9 at the concentrations higher than 200 μM (data not shown). Therefore, extrapolation of K_m values for glucuronidation by UGTs1A4 and 1A9 was performed with substrate concentrations ranging 5–100 μM for both isomers. However, no appreciable substrate inhibition was observed for glucuronidation of HO-TAM isomer s by UGT1A1. The kinetic parameters, V_{max}/K_m, obtained for the glucuronidation of HO-TAMs by the seven recombinant UGTs were in good accordance with their specific activities mentioned above (Tables 3 and 4). UGT2B15 showed the lowest K_m and highest V_{max}/K_m values for glucuronidation of *cis*-HO-TAM among the UGT isoforms that had considerable activity to this substrate (Table 4). Based on V_{max}/K_m, UGT2B15 showed the highest *cis* to *trans* ratio. *Cis*-HO-TAM was glucuronidated at considerably high V_{max} by UGTs1A1 and 1A4. However, their K_m values were 19- and 13-fold higher for UGTs1A1 and 1A4, respectively, than that observed for UGT2B15. These UGTs1A1 and 1A4 catalyzed the glucuronidation of *trans*-HO-TAM at higher V_{max} than did UGT2B15, so that the *cis* to *trans* ratio of V_{max}/K_m in glucuronidation of HO-TAM isomers was very low for these 1A enzymes.

3.4. Geometrical selectivity in sulfation of cis- and trans-HO-TAMs by recombinant human SULTs

A kinetic study using the four purified recombinant human SULTs, 1A1, 1A3, 1E1, and 2A1, indicated that the geometrical isomers of HO-TAM were predominantly sulfated by 1A1 at a *trans* to *cis* ratio of 21:1 based on k_{cat}/K_m (Table 5). SULT1E1 exhibited a very weak catalytic efficiency toward both HO-TAMs compared with 1A1. SULTs1A3 and 2A1 had no detectable activity toward

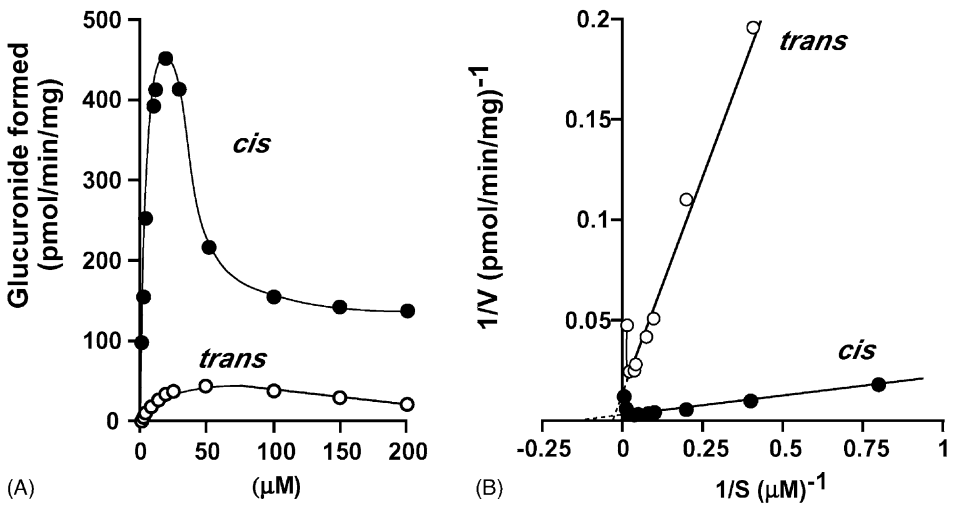

Fig. 6. Apparent reaction rates vs. concentrations of *cis*- and *trans*-HO-TAMs in glucuronidation by recombinant human UGT2B15 (A) and its double reciprocal plot (B). Panel (A): microsomes from insect cells expressing recombinant human UGT2B15 were incubated with various concentrations of *cis*-HO-TAM (●) and *trans*-HO-TAM (○) at 37° for 30–60 min in the presence of 2 mM [^{14}C-GA]UDPGA. The enzyme activities toward the geometrical isomers of HO-TAMs were determined as described in Section 2. Panel (B): double reciprocal plots of the data shown in panel (A). For determination of kinetic parameters, substrate concentrations ranging 1.25–12.5 μM for *cis*-HO-TAM and 1.25–50 μM for *trans*-HO-TAM, which did not show substrate inhibition, were used. Data are expressed as arithmetic mean values of at least three replicated incubations.

Table 4
Kinetic parameters for glucuronidation of *cis*- and *trans*-HO-TAMs by recombinant human UGTs expressed in insect cells

Human UGT	HO-TAMs						
	K_m (μM)		V_{max} (pmol/min/mg protein)		V_{max}/K_m		
	Cis	*Trans*	*Cis*	*Trans*	*Cis*	*Trans*	*Cis/trans*
1A1	138.1	129.4	291.0	166.0	2.1	1.3	1.6
1A3	N.D.[a]	N.D.					
1A4	94.8	101.1	314.6	546.8	3.3	5.4	0.6
1A6	N.D.	N.D.					
1A9	19.0	196.9	56.5	72.3	3.0	0.4	7.5
2B7	n.d.[b]	n.d.	n.d.	n.d.			
2B15	7.4	18.0	720.2	69.6	97.3	3.9	24.9

Cis- and *trans*-HO-TAMs were incubated in the presence of 2 mM [^{14}C-GA]UDPGA with microsomes (40–800 μg protein/mL) from insect cells expressing human UGTs, and radioactive glucuronides formed were separated and determined under the same conditions as described in Table 1. Kinetic parameters were obtained by double-reciprocal plots of substrate concentrations vs. reaction rates in the zero-order kinetics region as described in the text. Data are expressed as arithmetic mean values of at least three experiments.

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

[b] n.d.: not determined as enzyme activity was very low.

Table 5
Kinetic parameters for sulfation of *cis*- and *trans*-HO-TAMs by purified recombinant human SULTs

Human SULT	HO-TAMs							Specific activity (nmol/min/mg protein)[a]			
	k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (min^{-1} mM^{-1})			NP	E_2	DHEA	Dopamine
	Trans	*Cis*	*Trans*	*Cis*	*Trans*	*Cis*	*Trans/cis*				
1A1	3.95	0.34	20.0	37.1	197.5	9.2	21.5	558.3			
1A3	N.D.[b]	N.D.[b]									114.9
1E1	0.04	0.03	17.4	30.0	2.3	1.0	2.3		27.8		
2A1	N.D.[b]	N.D.[b]								257.3	

Cis- and *trans*-HO-TAMs were incubated for 10 min with purified recombinant human SULTs (0.1–6 μg protein/mL) in the presence of 10 μM [^{35}S]PAPS, and radioactive sulfates formed from HO-TAMs were separated by TLC and determined as described in Table 2. Kinetic parameters were obtained by double-reciprocal plots of substrate concentrations ranging from 5 to 200 μM vs. reaction rates in the zero-order kinetics region. Molecular weights of the recombinant human SULTs were counted as His-tagged proteins to calculate k_{cat} from V_{max} observed. The standard substrates for human SULTs, NP, E_2, DHEA, and dopamine were incubated only with the corresponding recombinant enzymes as described in Section 2. Data are expressed as arithmetic mean values of at least three experiments.

[a] Substrate concentrations used were 5 μM for NP, DHEA, and dopamine, and 20 μM for E_2. These substrates showed the strong substrate inhibition at concentrations higher than those described.

[b] N.D.: not detectable (less than 10 pmol/min/mg protein).

HO-TAMs, although these enzymes had high activity toward the typical substrates, dopamine and DHEA, respectively, as did 1A1 and 1E1 toward NP and E_2, respectively.

4. Discussion

No information has been available on geometrical selectivity in glucuronidation and sulfation of *cis*- and *trans*-HO-TAMs despite their importance as an estrogen agonist and an antagonist, respectively, as active metabolites of TAM, which is currently widely used for chemotherapy and chemoprevention of breast cancer [1]. The present study using microsomes and cytosol from human liver provided the first evidence that geometrical isomers of HO-TAM were *cis*- and *trans*-selectively glucuronidated and sulfated by microsomal UGTs and cytosolic SULTs, respectively (Tables 1 and 2). Our unpublished data indicated that the highly *cis*-selective microsomal glucuronidation and *trans*-selective cytosolic sulfation of HO-TAMs also proceeded in the liver of animals such as cynomolgus monkeys, Sprague–Dawley rats, ddy mice, and Beagle dogs. These data will be published elsewhere.

A glucuronide of *trans*-HO-TAM was reported by Lien and co-workers [27,28] to be excreted in the urine and bile of female humans and of male and female rats. It is probable that they simply considered that the aromatic hydroxylation product of the *trans*-stilbene derivative, TAM, was only *trans*-HO-TAM and also that the *trans*-HO-TAM metabolically formed was directly glucuronidated for excretion. Later, CYP-dependent geometrical inversion of *trans*-HO-TAM to *cis*-HO-TAM was demonstrated in human liver microsomes only in the presence of NADPH [7], and the existence of both *cis*- and *trans*-HO-TAMs was also demonstrated in breast carcinoma of TAM-resistant patients administered TAM [8,9].

Lien and co-workers [11,27] had likely failed to separate the urinary glucuronides of *cis*- and *trans*-HO-TAMs by HPLC, even if both glucuronides were present in their samples. Actually, we could not separate the glucuronides formed from *cis*- and *trans*-HO-TAMs by human liver microsomes using the same solvent system as they reported for elution of the octadecylsilica column. Under their HPLC conditions, a mixture of both glucuronides was eluted as an inseparable single peak from the reverse phase partition column (data not shown). A more polar solvent mixture should have been used for such separation as demonstrated in the present study using a gradient system that controlled the polarity of the mobile phase (Fig. 2A and B). β-Glucuronidase should have been used for further identification of their glucuronide as shown in the present study. The aglycons, *cis*- and *trans*-HO-TAMs, were also separable by HPLC (Fig. 2C and D).

Human UGTs with calculated molecular masses of 59–61 kDa, based on molecular cloning, are bound to endoplasmic reticulum in glycosylated form, so that purification of the enzymes to homogeneity with the proper activity has been difficult [29,30]. Therefore, the use of microsomes could be the best way to estimate the geometrical selectivity in the hepatic glucuronidation of *cis*- and *trans*-HO-TAMs in human liver and insect cells expressing human UGTs separately.

Recent molecular cloning studies have demonstrated that there exist at least 17 UGT mRNAs in the human, and that they are divided into two families, UGT1 and UGT2, consisting of 9 and 8 members, respectively, on the basis of amino acid sequence identity [31–33]. It is known that five isoforms of the UGT1A subfamily, UGTs1A1 [34,35], 1A3 [36], 1A4 [34], 1A6 [37], and 1A9 [38], and seven isoforms of the UGT2B subfamily, UGTs2B4 [39–41], 2B7 [41–44], 2B10 [40], 2B11 [45], 2B15 [46,47], 2B17 [48,49], and 2B28 [33], are expressed in the human liver. These UGTs have been cloned and heterologously expressed with enzyme activity. However, only three isoforms have been purified from human liver microsomes as native homogeneous proteins [50,51]. Of these three purified human UGTs, only one was identified with molecularly cloned UGT2B15 [46] by N-terminal amino acid sequence analysis of the enzyme protein [52].

Human UGTs have been demonstrated by using insect cell microsomes separately containing the recombinant enzymes to show very broad substrate specificity to various xenobiotic phenols, alcohols, and carboxylic acids except for bilirubin for UGT1A1 and morphine for UGT2B7 as probe substrates [53,54].

Cis-HO-TAM was a good substrate for recombinant human UGT2B15 compared with the other six tested human UGTs (Tables 3 and 4). UGT2B15 is recognized to readily catalyze the glucuronidation of bulky hydrophobic substrates such as dihydrotestosterone, 5-androstane-3α,17β-diol, flavones, and HO-BP [46,55]. *Cis*-HO-TAM may be a new member to be grouped into these UGT2B15 substrates. Support may be provided by the observed good correlation between HO-BP and *cis*-HO-TAM glucuronidations, which both proceeded at considerably high rates by liver microsomes from 10 human subjects (Fig. 3A). However, despite its hydrophobicity and bulkiness almost equal to those of *cis*-HO-TAM (Fig. 1), *trans*-HO-TAM was a much poorer substrate for UGT2B15 than the *cis*-isomer. In human hepatic microsomes, UGT2B15 may play an important role in the *cis*-selective glucuronidation of HO-TAM isomers, because *cis*-HO-TAM glucuronidation both by microsomes from human liver and from insect cells expressing UGT2B15 was strongly inhibited by the substrate at concentrations higher than 25 μM (Figs. 4A–C and 6A). However, at least another UGT catalyzing the *cis*-selective glucuronidation of the HO-TAM isomers with high efficiency is likely to exist in human liver microsomes, because the ratio of *cis*- to *trans*-HO-TAM glucuronidation by human liver microsomes was much higher than that by insect cell microsomes containing UGT2B15 (Tables 1 and 3). It should be noted that we have no evidence for the role of human hepatic UGTs2B4, 2B10, 2B11, 2B17, and 2B28, all of which were commercially unavailable and were not tested in this study.

A recent study reported by Lévesque *et al.* [56] on the genetic polymorphism of human UGT2B15 might be correlated in part with the observed large individual difference in the hepatic microsomal glucuronidation of *cis*-HO-TAM. They demonstrated that human UGT2B15 occurred in two polymorphic forms having aspartic acid (D) or tyrosine (Y) as an amino acid residue at position 85 and also that recombinant UGT2B15(Y^{85}) had activity twice higher than did recombinant UGT2B15(D^{85}). A population study of human *UGT2B* genes by Lampe *et al.* [57] indicated that 47 and 19% of Asians were D^{85}/D^{85} and Y^{85}/Y^{85}, respectively, whereas 22 and 32% of Caucasians were D^{85}/D^{85} and Y^{85}/Y^{85}, respectively.

SULT1A1, one of four major SULTs occurring in the human liver, was strongly suggested in this study to play the most important role among the four SULTs in the hepatic *trans*-selective sulfation of the geometrical isomers of HO-TAM. In contrast to the human hepatic microsomal *cis*-selective glucuronidation of HO-TAM isomers, their sulfation by cytosol from the same human livers proceeded in a *trans*-selective manner with a little individual difference; the difference was approximately 2-fold between the lowest and highest hepatic *trans*-HO-TAM-sulfating activities in 10 humans (Table 2). This individual difference is in good accordance with the previous demonstration by Ozawa *et al.* [58] that hepatic levels of mRNA for SULT1A1 differed about 2-fold between the lowest and highest mRNA levels in 26 Caucasians. However, unlike this study, they also demonstrated that NP-sulfating activity differed approximately 6-fold in these subjects. As to this discrepancy between the hepatic SULT1A1 mRNA levels and NP-sulfating activities, they suggested that

hepatic SULTs other than 1A1 might participate in the sulfation of NP in the liver. The 6-fold difference in the hepatic NP-sulfating activity has also been demonstrated by Raftogianis *et al.* [59] in 61 human subjects. A reported significant ethnic difference in gene frequency of the polymorphic *SULT1A1* alleles between Caucasians and Chinese [60] might be correlated with the difference in the hepatic NP-sulfating activities between Caucasians and 10 Japanese subjects tested in this study. However, no information on the variability of SULT activities has been reported in Japanese population. It should be noted that we have no evidence for the role of three minor human hepatic SULTs, 1A2 [21], 1B1 [61], and 1C1 [62] in their *trans*-selective sulfation of HO-TAM isomers.

The lack of correlation between sulfation and glucuronidation of HO-TAMs in human livers suggests that expression levels of SULTs and UGTs active toward these substrates are independently regulated in the human liver. A similar result has been reported by Pacifici *et al.* [63] in cytosolic sulfation and microsomal glucuronidation of 2-naphthol in fetal and adult human livers. They demonstrated that the rate of 2-naphthol sulfation correlated with that of its glucuronidation in 34 fetal livers, whereas no relationship was observed between the activity of SULT and UGT in 27 adult livers, suggesting that these enzymes were under a common developmental pattern in fetus, but were independently regulated in postnatal development.

The present study suggests that it may be of clinical importance to determine the levels of UGT and SULT activities toward *cis*- and *trans*-HO-TAMs in the breast tumor tissue of patients before and during their treatment with TAM in order to estimate whether TAM is effective or not for them. This suggestion is based on the aforementioned fact that in the breast tumor tissue from TAM-resistant patients, the estrogenic metabolite *cis*-HO-TAM, is found at a higher concentration than the anti-estrogenic metabolite, *trans*-HO-TAM [8,9].

Thus, the present study strongly suggests that in the human liver, the anti-estrogenic active metabolite, *trans*-HO-TAM, formed from the antibreast cancer drug, TAM, by CYPs is sulfated mainly by SULT1A1 for excretion and also that the estrogenic metabolite, *cis*-HO-TAM, formed from its *trans*-isomer by the geometrical inversion with CYPs in the presence of NADPH, is glucuronidated for excretion by UGTs, including 2B15 as a potential major candidate.

A study on the survey of the unknown *in vivo* metabolites, *trans*-HO-TAM sulfate and *cis*-HO-TAM glucuronide, in the human is now in progress in our laboratory.

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