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Quaternary ammonium-linked glucuronidation of tamoxifen by human liver microsomes and UDP-glucuronosyltransferase 1A4

Teppei Kaku[a], Kenichiro Ogura[a], Takahito Nishiyama[a], Tomokazu Ohnuma[a], Kei Muro[b], Akira Hiratsuka[a,*]

[a]*Department of Drug Metabolism and Molecular Toxicology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji-shi, Tokyo 192-0392, Japan*
[b]*Division of GI Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Tokyo 104-0045, Japan*

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Abstract

Tamoxifen (TAM), a nonsteroidal antiestrogen, is the most widely used drug for chemotherapy of hormone-dependent breast cancer in women. In the present study, we found a new potential metabolic pathway of TAM via *N*-linked glucuronic acid conjugation for excretion in humans. TAM N^+-glucuronide was isolated from a reaction mixture consisting of TAM and human liver microsomes fortified with UDP-glucuronic acid (UDPGA) and identified with a synthetic specimen by high-performance liquid chromatography-electrospray ionization-mass spectrometry. However, no TAM-glucuronidating activity was detected in microsomes from rat, mouse, monkey, dog, and guinea pig livers. A strong correlation ($r^2 = 0.92$) was observed between *N*-glucuronidating activities toward TAM and trifluoperazine, a probe substrate for human UDP-glucuronosyltransferase (UGT) 1A4, in human liver microsomes from eight donors (five females, three males). However, no correlation ($r^2 = 0.02$) was observed in the activities between 7-hydroxy-4-(trifluoromethyl)coumarin and TAM. Only UGT1A4 catalyzed the *N*-linked glucuronidation of TAM among recombinant UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in insect cells. Apparent K_m values for TAM *N*-glucuronidation by human liver microsomes and recombinant UGT1A4 were 35.8 and 32.4 μM, respectively. These results strongly suggested that UGT1A4 could play a role in metabolism and excretion of TAM without Phase I metabolism in human liver. TAM N^+-glucuronide still had binding affinity similar to TAM itself for human estrogen receptors, ERα and ERβ, suggesting that TAM N^+-glucuronide might contribute to the biological activity of TAM in vivo.

Keywords: Tamoxifen; UDP-glucuronosyltransferase; *N*-Glucuronidation; Human liver microsomes; Estrogen receptor; Antiestrogen

1. Introduction

Tamoxifen (TAM, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene) is a nonsteroidal triphenylethylene antiestrogen that has been widely used for chemotherapy and chemoprevention of breast cancer, one of the major causes of cancer-related death in women [1]. The antiestrogenic activity of TAM is based on competing activity with β-estradiol for estrogen receptors (ERs). However, TAM is not a pure antiestrogen and has agonistic properties in some estrogen target tissues [2]. The paradoxical activity of TAM remains unclear. Also, TAM was shown to have 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, 4-hydroxytamoxifen (4-HO-TAM), *N*-desdimethyl-TAM, 4-hydroxy-*N*-desmethyl-TAM, TAM *N*-oxide and the primary alcohol named metabolite Y [4–7]. Among these Phase I metabolites, 4-HO-TAM has been considered to be

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

* Corresponding author. Tel.: +81-426-76-4516; fax: +81-426-76-4517.
E-mail address: hiratuka@ps.toyaku.ac.jp (A. Hiratsuka).

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an active metabolite of TAM because of its higher affinity toward ERs than the parent drug and other side-chain metabolites [8]. Interestingly, 4-HO-TAM was partially converted in vivo to its geometrical isomer, *cis*-4-HO-TAM, a much less potent antiestrogen that 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 in the manner of *cis* ⩾ *trans* by human liver microsomes and sulfated in the manner of *trans* ⩾ *cis* by human liver cytosol [10]. We also demonstrated through the use of recombinant enzymes that such sulfation and glucuronidation were catalyzed by human sulfotransferase isoform SULT1A1 and UDP-glucuronosyltransferase (UGT) isoform UGT2B15, respectively [10].

A pharmacokinetic study in humans showed that the major route of TAM excretion was via the feces [11]. In females, more than 60% of the administered radiolabeled TAM was excreted as unchanged drug, with most of the remaining fecal radioactivity due to Phase II conjugated metabolites. Only 9–14% of radiolabeled TAM was eliminated into urine. Lien et al. [12] 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, 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. However, treatment of the fecal extract from one patient with β-glucuronidase increased the concentration of TAM and TAM metabolites, indicating the existence of glucuronic acid conjugates [12]. They also noted that significant amounts of ''conjugated TAM,'' which released TAM by treatment of β-glucuronidase, could be recovered from bile, although they provided no further information on conjugated TAM.

Entero-hepatic circulation of unmetabolized TAM as well as hydroxylated TAM metabolites in patients who were administered TAM was observed [11]. Therefore, we hypothesized a possible excretion pathway of TAM whereby TAM could be excreted into bile via TAM N^+-glucuronide. *N*-Glucuronidation has been known to produce *N*-linked glucuronide as urinary and biliary metabolites for more than 30 nitrogen-containing drugs and chemicals [13]. In particular, *N*-glucuronidation has been extensively studied in the metabolism of H_1 antihistamine and antidepressant drugs with an aliphatic tertiary amine group, such as chlorpheniramine, diphenhydramine, ketotifen, amitriptyline, and imipramine [14–17]. Luo et al. [14] reported fecal excretion of cyclizine N^+-glucuronide and suggested possible entero-hepatic circulation of the drug via N^+-glucuronide metabolites. However, although TAM has an *N*,*N*-dimethylaminoalkyl side chain in its structure, there is no information on *N*-glucuronidation of TAM and TAM metabolites.

In the present study, we investigated whether human liver microsomes and recombinant UGT isoforms were capable of catalyzing *N*-glucuronidation of TAM to reveal a new potential excretion pathway of the anti-cancer drug. Binding affinity of TAM N^+-glucuronide to human estrogen receptors, ERα and ERβ, was also investigated.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), β-estradiol, eugenol, β-glucuronidase (Type VII-A from *Escherichia coli*, 100 U/ml), D-saccharic acid 1,4-lactone, TAM and UDP-glucuronic acid (UDPGA) were purchased from Sigma Chemicals Co. 7-Hydroxy-4-(trifluoromethyl)coumarin (HFC) was purchased from Kanto Chemicals Co. Alamethicin, trifluoperazine and [^{14}C]UDPGA (300 mCi/mmol) were purchased from ICN Pharmaceuticals, Inc. Microsomes prepared from insect cells expressing recombinant human UGT1A1, UGT1A3, UGT1A6 and UGT2B7 in Sf9 cells were purchased from PanVera Co., and recombinant human UGT1A4, UGT1A9, UGT2B4, UGT2B15, and UGT2B17 expressed in BTI-TN-5B1-4 cells were purchased from Gentest Co. 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) using a reverse phase column (Capcell Pak C_{18} AQ, 2.0 mm × 150 mm, 5 μm particles) (Shiseido). The mobile phase was composed of methanol and 100 mM ammonium acetate buffer, pH 5.0. Separation was carried out by a linear gradient from 75 to 100% methanol in 15 min at a flow rate of 0.2 ml/min. The mass spectrometer used was a Micromass model LCT. For a positive ESI-TOF-MS spectrum of synthetic TAM N^+-glucuronide, the sample was 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. NMR spectroscopy

The 500 MHz ^{1}H NMR spectra were recorded on a Bruker model DRX500 at 300 K. The synthetic TAM N^+-glucuronide was dissolved in dimethyl sulfoxide-d_6 to a concentration of 1 mg/0.5 ml.

2.4. Chemical synthesis of TAM N^+-glucuronide

Methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate (160 mg, 0.4 mmol) and TAM (100 mg, 0.27 mmol) were dissolved in dichloromethane (0.2 ml) 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 0.3 ml of 0.5 M aqueous sodium bicarbonate and stirred for 12 h at room temperature to hydrolyze protecting acetyl groups and carboxyl methyl ester. After the addition of water (25 ml), the mixture was extracted three times with the same volume of ether to remove unreacted TAM. The aqueous layer was adjusted with 0.1N HCl to pH 5.0 and loaded onto a preparative C_{18} column (1 cm × 5 cm, 55–105 μm, Millipore), which was pre-equilibrated with water. Unbound inorganic salts were washed out with water, and the bound TAM N^+-glucuronide was eluted with methanol. Evaporation of the methanolic eluate yielded 11.3 mg of white powder (6.7%). ^{1}H NMR (500 MHz, dimethyl sulfoxide-d_6): δ 0.85 (t, 3H, *J* 7.4 Hz, CH_2CH_3), 2.37 (q, 2H, *J* 7.4 Hz, CH_2CH_3), 3.12 (s, 3H, N–CH_3), 3.15 (m, 1H, H-4′), 3.18 (s, 3H, N–CH_3), 3.27 (m, 1H, H-3′), 3.40 (d, 1H, *J* 10.1 Hz, H-5′), 3.54 (m, 1H, H-2′), 3.77–3.87 (m, 2H, N–CH_2CH_2–O), 4.35 (br.t, 2H, *J* 5.0 Hz, N–CH_2CH_2–O), 4.64 (d, 1H, *J* 8.8 Hz, H-1′), 5.45 (bs, 1H, OH-3′), 5.97 (bs, 1H, OH-2′), 6.68 (d, 2H, *J* 8.7 Hz, ArH, *ortho* to NCH_2CH_2O–), 6.77 (d, 2H, *J* 8.7 Hz, ArH, *meta* to NCH_2CH_2O–), 7.08 (bs, 1H, OH-4′), 7.11–7.40 (m, 10H, ArH). ^{13}C NMR (125.7 MHz, dimethyl sulfoxide-d_6): δ 13.27, 28.56, 47.99, 49.13, 61.25, 61.95, 70.03, 71.37, 76.05, 77.27, 94.61, 113.70, 126.25, 126.68, 127.97, 128.28, 128.92, 129.33, 131.31, 135.66, 137.77, 140.93, 141.71, 143.18, 155.45, 169.97. ESI-TOF-MS *m/z* (relative intensity): 548.2629 $[M]^+$ (100%), 372.2281 (33%). Calculated mass is 548.2648 for $C_{32}H_{38}NO_7$ $[M]^+$.

2.5. Human liver samples

Liver samples from eight cancer patients (five females, three 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 human 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 as follows: 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 60 min 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 expressing UGT isoforms toward TAM, trifluoperazine, eugenol, and HFC were carried out as described previously [20] with the following modifications. Human liver microsomes were activated with alamethicin (50 μg/mg protein) for 15 min in an ice bath as described by Fisher et al. [21]. This step was omitted for the insect cell microsomes, because alamethicin did not affect glucuronidating activity of insect microsomes. 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 [^{14}C]UDPGA (0.25 μCi) in a final volume of 50 μl. The reaction was started by the addition of the substrate (dissolved in 2.5 μl dimethyl sulfoxide) and terminated by the addition of 50 μl of methanol. After centrifugation at 12,000 × *g* for 5 min, 30 μl of the supernatant was applied onto a silica gel 60 F_{254} thin layer plate (Merck Ltd.) having a preabsorbent sample-spotting area, and developed with *n*-butanol/acetone/glacial acetic acid/30% (w/v) ammonia/water (70:50:9:8:60, v/v/v/v). The radioactivity of the glucuronide was measured by radioluminography with a BAS 2000 bioimaging analyzer (Fuji Photo Films Co., Ltd.). The addition of the β-glucuronidase inhibitor, D-saccharic acid 1,4-lactone (1 mM), had no effect on UGT activity. The concentration (5%, v/v) of dimethyl sulfoxide did not affect the UGT activities of human and insect microsomes toward all substrates used in the present study. Determinations of TAM *N*-glucuronidating activity of liver microsomes from rats, mice, monkeys, dogs, and guinea pigs 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 glucuronidation of TAM, substrate concentrations ranging from 10 to 400 μM were used. The kinetic constants were determined by extrapolation from Michaelis–Menten plots.

2.8. Identification of TAM N^+-glucuronide formed by human liver microsomes in the presence of UDPGA

For identification of TAM N^+-glucuronides formed by human liver microsomes, the reaction was performed on a large scale for 2 h. The final volume of the reaction mixture was increased up to 2 ml, and the TAM concentration was 0.4 mM. The reaction was started by the addition of the

substrate (dissolved in 100 μl of dimethyl sulfoxide) and terminated by the addition of 2 ml of methanol. After the termination, the reaction mixture was centrifuged at 2000 × *g* for 10 min. The supernatant was lyophilized, and the residue was dissolved with 200 μl methanol for the analysis by HPLC-ESI-TOF-MS as indicated above. 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 TAM, TAM N^+-glucuronide, and diethylstilbestrol (DES) to β-estradiol for human ERα and ERβ were assayed using a Ligand Screening System (TOYOBO Co., Ltd.). Briefly, purified recombinant human ERα or ERβ was incubated in a microplate with various concentrations of these ligands in the presence of β-estradiol (12.5 nM) at 4 °C for 1 h. After incubation, unbound β-estradiol was allowed to compete with anti-β-estradiol antibody and horseradish peroxidase (HRP)-labeled β-estradiol at 4 °C for 1 h. After washing the plate, the remaining peroxidase-labeled β-estradiol bound on the well was measured by densitometry using a microplate reader Model SAFIRE (Tecan Japan Co., Ltd.) 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 β-estradiol binding by 50%) to that of DES. Data were expressed as the arithmetic mean values ± S.D. obtained from at least three replicated assays.

3. Results

3.1. Chemical synthesis of TAM N^+-glucuronide

A simple reaction of TAM with methyl(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide)uronate in dichloromethane gave the *N*-linked glucuronide. NMR spectrum of the glucuronide showed the signal for the anomeric proton on the sugar ring at δ 4.64 ppm as a doublet with a coupling constant of 8.8 Hz. The chemical shift and coupling constant were characteristic of N^+-linked β-glucuronides [13,14,17]. Separation of *N*,*N*-dimethyl proton signals as singlets at δ 3.12 and 3.18 ppm indicated that these methyl groups were unequivalent due to the formation of the fourth N–C bond.

3.2. Identification of TAM N^+-glucuronide formed by human liver microsomes

Enzymatic formation of TAM N^+-glucuronide by human liver microsomes was first characterized by HPLC (Fig. 1)

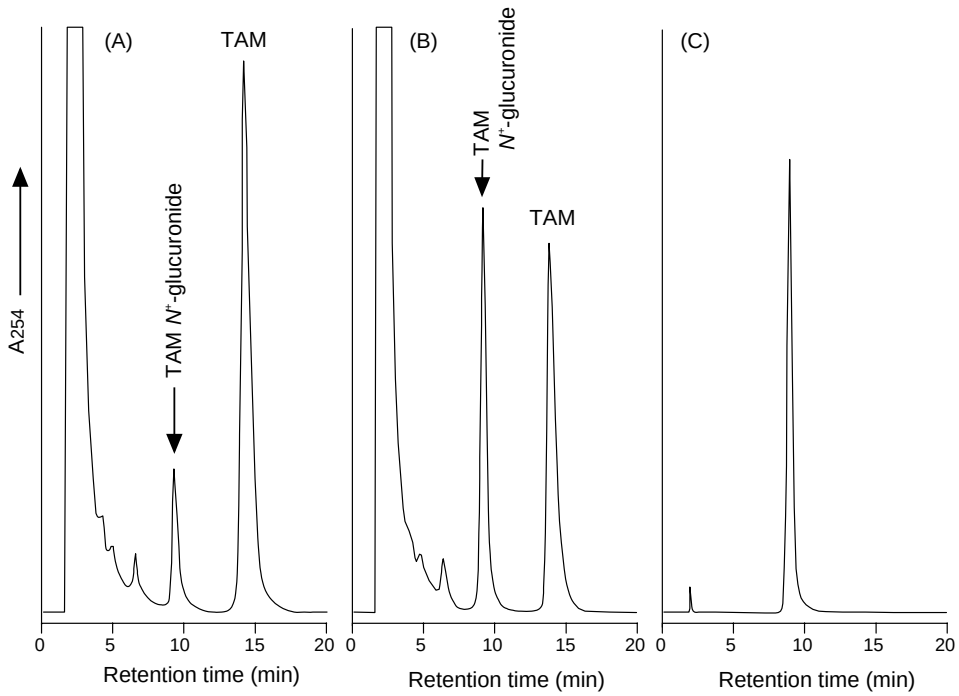

Fig. 1. HPLC separation of TAM and TAM N^+-glucuronide formed by human liver microsomes and recombinant UGT1A4. Human liver microsomes (from donor F4 in Table 1) (A) or recombinant UGT1A4 (B) was incubated at 37 °C for 2 h with 0.4 mM TAM in the presence of 2 mM UDPGA. Details are described in the text. Chromatogram C represents the elution profile of synthetic TAM N^+-glucuronide.

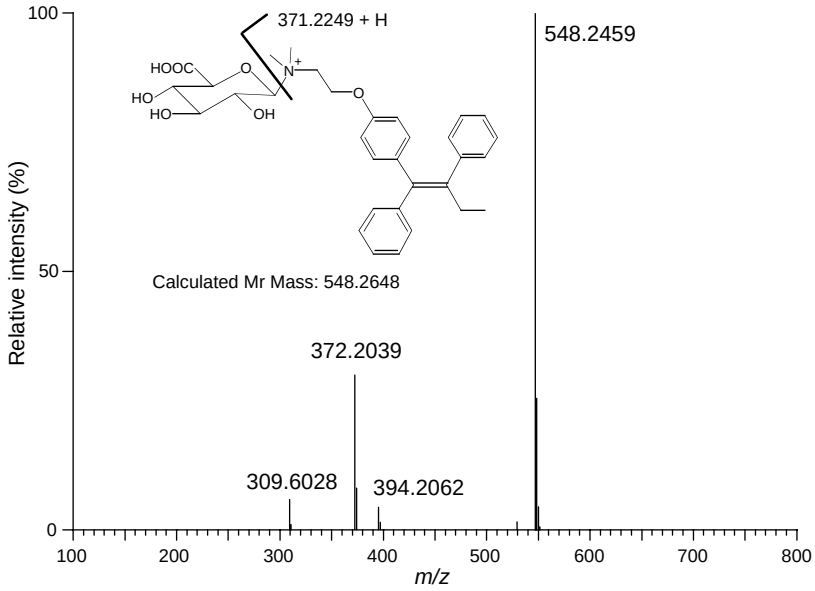

Fig. 2. Representative mass spectrum of TAM N^+-glucuronides formed from TAM by human liver microsomes. The spectrum was taken at the retention time of 9.4 min in Fig. 1A.

and HPLC-ESI-TOF-MS (Fig. 2). Fig. 1 shows representative HPLC chromatograms of enzymatically formed TAM N^+-glucuronide by human microsomes and recombinant human UGT1A4 in the presence of UDPGA. The TAM N^+-glucuronide formed in the reaction mixture had a retention time of 9.4 min (chromatograms A and B), which was identical to that of the synthetic specimen (chromatogram C). Treatment with β-glucuronidase of the N^+-glucuronide eluted from the HPLC column afforded TAM, which was identified by HPLC-MS (data not shown). MS analysis of the eluted TAM N^+-glucuronide showed a mass spectrum (Fig. 2) very similar to that of the synthetic specimen. The mass spectrum showed an $[M^+]$ ion at *m/z* 548.2459, corresponding to TAM N^+-glucuronide and a fragment ion at *m/z* 372.2039, corresponding to the parent drug TAM + H with loss of the glucuronic acid moiety (176 amu).

3.3. TAM N-glucuronidation by human liver microsomes

The rates of *N*-linked glucuronide formation of TAM were determined using liver microsomes from eight donors (five females, F1–F5; three males, M1–M3). Pretreatment of human liver microsomes with alamethicin increased activity approximately 36%, while the addition of Triton X-100 (400 μg/mg protein) into the reaction mixture rather inhibited activity. As shown in Table 1, TAM *N*-glucuronidating activity varied, ranging from 23.2 to 40.4 pmol/min/mg protein. The activities toward standard substrates, trifluoperazine and HFC, also varied among the liver microsomes from the eight donors, ranging from 50.8 to 72.2 pmol/min/mg and from 2757 to 5475 pmol/min/mg, respectively. Correlation analysis showed a very strong correlation ($r^2 = 0.92$) between TAM *N*-glucuronidating activity and trifluoperazine *N*-glucuronidating activity (Fig. 3A). In contrast, no correlation ($r^2 = 0.02$) was observed between HFC *O*-glucuronidation and TAM *N*-glucuronidation (Fig. 3B). In addition, there was no correlation ($r^2 = 0.01$) between trifluoperazine and HFC conjugating activities.

3.4. TAM N-glucuronidation by human UGT isoforms expressed in insect cells

Recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4,

Table 1
N-Glucuronidation of TAM by human liver microsomes

Donor	Sex	Age	Specific activities (pmol/min/mg protein)		
			TAM	Trifluoperazine	HFC
F1	F	71	27.0 ± 4.5	59.9 ± 5.8	2757 ± 163
F2	F	46	39.1 ± 3.1	71.3 ± 6.4	3917 ± 189
F3	F	29	32.0 ± 2.7	66.6 ± 7.1	4440 ± 248
F4	F	50	36.0 ± 7.2	68.0 ± 2.1	4074 ± 192
F5	F	52	40.4 ± 1.6	72.2 ± 6.4	2940 ± 123
M1	M	54	37.3 ± 5.1	67.1 ± 7.0	5475 ± 251
M2	M	56	36.1 ± 2.1	69.5 ± 4.1	4942 ± 161
M3	M	67	23.2 ± 1.8	50.8 ± 2.5	4104 ± 105

TAM (100 μM) was incubated for 1 h with human liver microsomes from five females (F1–F5) and three males (M1–M3) 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 $MgCl_2$. Trifluoperazine (200 μM) and HFC (50 μM) were used as standard substrates for determining microsomal activity under the same conditions as stated above except for incubation time (30 min) for HFC. 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.

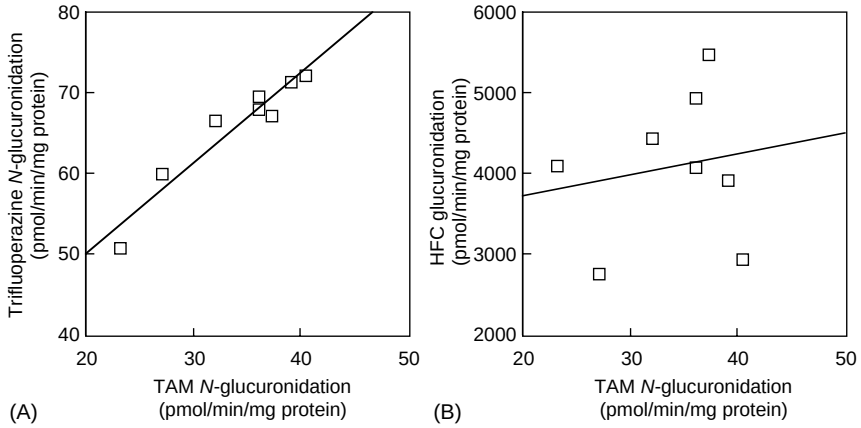

Fig. 3. Correlation between glucuronidation of TAM and trifluoperazine or HFC by human liver microsomes from eight donors. Glucuronidating activities from eight donors (F1–F5 and M1–M3) toward TAM, trifluoperazine and HFC listed in Table 1 were used. Correlation efficiencies were as follows: Panel A, TAM- and trifluoperazine-glucuronidating activities ($r^2 = 0.92$) and Panel B, TAM- and HFC-glucuronidating activities ($r^2 = 0.02$).

UGT2B7, UGT2B15, and UGT2B17) independently expressed in insect cells were examined for their ability to catalyze glucuronidation of TAM in comparison with activity toward the reference substrates, trifluoperazine and HFC. Of these recombinant human enzymes, only UGT1A4 showed TAM and trifluoperazine *N*-glucuronidating activity (Table 2). In contrast, all UGT isoforms except for UGT1A4 exhibited glucuronidating activity toward HFC, which was measured according to the manufacturer's instructions using the indicated substrates.

3.5. Species difference in liver microsomal TAM N-glucuronidation

None of the liver microsomes from the experimental animals showed detectable TAM *N*-glucuronidating activity (less than 5 pmol/min/mg protein), whereas these microsomes showed to have HFC-glucuronidating activity (nmol/min/mg protein) of 2.6 ± 0.2 (male rat), 2.5 ± 0.3 (female rat), 3.4 ± 0.7 (male mice), 4.0 ± 0.6 (male monkeys), 9.9 ± 0.8 (male dogs), and 8.9 ± 0.6 (male guinea pigs) at the substrate concentration of 50 μM.

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

Kinetic analysis of TAM *N*-glucuronidation was performed using human liver microsomes (from donor F4) and recombinant UGT1A4. Similar apparent K_m values were observed for TAM *N*-glucuronidation by human microsomes (35.8 ± 6.0 μM) and recombinant UGT1A4 (32.4 ± 4.5 μM). The insect cell microsomes containing recombinant UGT1A4 catalyzed TAM *N*-glucuronidation with a V_{max} value of 401.5 ± 20.8 pmol/min/mg protein which was approximately 4.5-fold higher than that of human microsomes (89.3 ± 12.5 pmol/min/mg protein).

Table 2
N-Glucuronidation of TAM by recombinant human UGTs expressed in insect cells

Human UGT isoform	Specific activities (pmol/min/mg protein)		
	TAM	Trifluoperazine	HFC
UGT1A1	N.D.[a]	N.D.	242.3 ± 15.3
UGT1A3	N.D.	N.D.	56.5 ± 1.5
UGT1A4	218.2 ± 5.8	504.3 ± 10.6	N.D.
UGT1A6	N.D.	N.D.	886.0 ± 76.5
UGT1A9	N.D.	N.D.	1972.1 ± 127
UGT2B4	N.D.	N.D.	379.4 ± 10.2
UGT2B7	N.D.	N.D.	361.0 ± 18.5
UGT2B15	N.D.	N.D.	287.6 ± 11.5
UGT2B17	N.D.	N.D.	31.9 ± 9.2 (409.0 ± 10.1)[b]

TAM (100 μM) was incubated for 1 h with insect 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 $MgCl_2$. Trifluoperazine (200 μM) and HFC (50 μM) were used as standard substrates for determining microsomal activity under the same conditions as stated above except for incubation time (30 min) for HFC. Activity toward eugenol (200 μM) was also determined as the indicated substrate for UGT2B17 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] Not detectable (less than 5 pmol/min/mg protein).

[b] Activity toward eugenol.

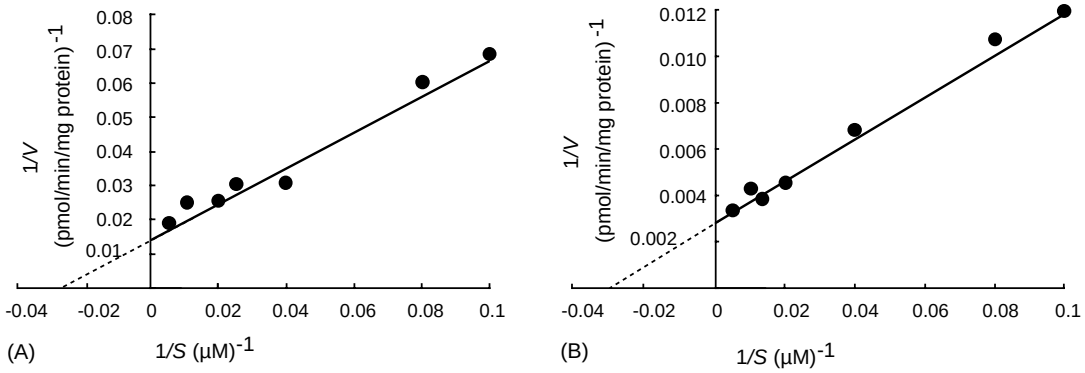

Fig. 4. Double reciprocal plot for *N*-linked glucuronidation by human liver microsomes and recombinant UGT1A4. Liver microsomes from donor F4 (Panel A) or recombinant UGT1A4 (Panel B) were incubated with various concentrations of TAM at 37 °C for 1 h in the presence of 2 mM [^{14}C]UDPGA. For determination of kinetic parameters, substrate concentrations ranging 10–400 μM were used. Data are expressed as arithmetic mean values of at least three replicated incubations.

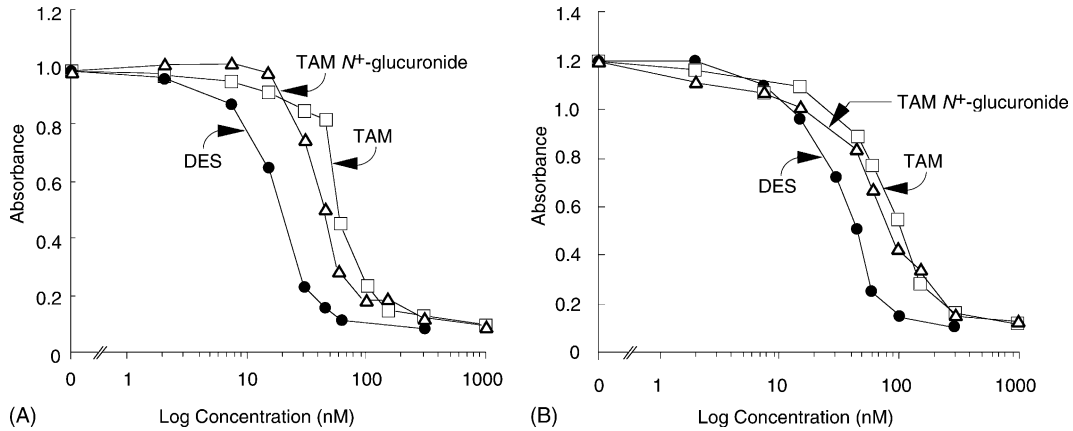

Fig. 5. Competitive binding affinities of DES, TAM, and TAM N^+-glucuronide for ERα and ERβ. Purified recombinant human ERα (Panel A) or ERβ (Panel B) was incubated with various concentrations of DES (●), TAM (□), and TAM N^+-glucuronide (△) in the presence of β-estradiol (12.5 nM) at 4 °C for 1 h. The amount of unbound β-estradiol was assayed by absorptiometry based on competition with HRP-labeled β-estradiol to anti-β-estradiol antibody as described in the text.

No substrate inhibition was observed at the substrate concentrations used (Fig. 4).

3.7. Binding affinity of TAM N^+-glucuronide for human ERs

The binding affinities of TAM and TAM N^+-glucuronide 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 β-estradiol and ERs is reduced to 50% of binding in the absence of a competing ligand, TAM and TAM N^+-glucuronide had similar affinities for both ERs (Fig. 5). Among the chemicals tested, DES competed with β-estradiol at the lowest IC_{50} values of 18.3 ± 1.0 nM and 31.4 ± 0.9 nM for human ERα and ERβ binding, respectively. TAM and TAM N^+-glucuronide competed at similar concentrations of 57.8 ± 1.6 nM and 47.6 ± 1.8 nM for ERα and 89.7 ± 3.1 nM and 80.8 ± 2.7 nM for ERβ, respectively. The relative binding affinity of DES, TAM, and TAM N^+-glucuronide, taken at the ratio of IC_{50} values to that of DES, were 100, 31.7, and 38.4 for ERα and 100, 35.0, and 38.9 for ERβ, respectively.

4. Discussion

The present study provides the first evidence of TAM *N*-glucuronidation in human liver microsomes in vitro. TAM N^+-glucuronide formed in the reaction mixture consisting of human liver microsomes and TAM in the presence of UDPGA was identified with the synthetic specimen by HPLC-ESI-TOF-MS. Substrate specificity of nine isoforms of human hepatic UGTs indicated that *N*-glucuronidation in human liver microsomes was catalyzed only by UGT1A4. Also revealed was that the activity of TAM *N*-glucuronidation of human liver microsomes from eight individuals strongly correlated with activity to trifluoperazine, which was known to be a probe substrate for UGT1A4 [22]. The similar K_m values for *N*-glucuronidation

by human liver microsomes (35.8 μM) and by recombinant UGT1A4 (32.4 μM) also indicated that UGT1A4 was responsible for human hepatic microsomal *N*-glucuronidation of TAM. The higher V_{max} value for TAM *N*-glucuronidation by recombinant UGT1A4 indicated that the baculovirus expression system, used for the expression of UGT1A4, could produce a larger amount of UGT1A4 protein in the insect cell microsomes than that existed in human liver microsomes.

At least 17 UGT mRNAs are known to exist in the human. These are divided into two families, UGT1 and UGT2, consisting of nine and eight isoforms, respectively, on the basis of amino acid sequence identity. Among these isoforms, five isoforms of the UGT1A subfamily, UGT1A1 [23,24], UGT1A3 [25], UGT1A4 [23], UGT1A6 [26], and UGT1A9 [27], and seven isoforms of the UGT2B subfamily, UGT2B4 [28–30], UGT2B7 [29,31–33], UGT2B10 [30], UGT2B11 [34], UGT2B15 [35,36], UGT2B17 [37,38], and UGT2B28 [39], are known to be expressed in the human liver. Therefore, we used these five isoforms of the UGT1A subfamily and four of the isoforms of the UGT2B subfamily, which are currently commercially available. It should be noted that we have no evidence of a role in TAM *N*-glucuronidation by human hepatic UGT2B10, UGT2B11, and UGT2B28, all of which were commercially unavailable and were not tested in this study.

N-Glucuronidation of primary, secondary, and tertiary amines are known to be catalyzed mainly by UGT isoforms, UGT1A3 and/or UGT1A4. *N*-Glucuronidation of antihistamic and antidepressant drugs, clozapine, chlorpromazine, loxapine, amitriptyline, imipramine, and (*R*)- and (*S*)-ketotifens were identified to be catalyzed by both isoforms UGT1A3 and UGT1A4 [40–42]. Known K_m values for *N*-glucuronidation by UGT1A4 of these drugs are 27, 93, 98, 100, 310, and 59, 85 μM, respectively. However, these drugs are poor substrates for UGT1A3 (K_m values ranged from 96 to 514 μM). Only trifluoperazine has been shown to be a probe substrate specific for UGT1A4 [22]. In the present study, TAM was conjugated to form a quaternary ammonium-linked glucuronide only by UGT1A4, with a K_m value of 32.4 μM. The data presented here shows that TAM is specifically conjugated by UGT1A4, and TAM can be a new probe substrate for this isoform.

Liver microsomes from Sprague–Dawley rats, ddy mice, cynomolgus monkeys, beagle dogs, and Hartley guinea pigs failed to produce detectable amount of TAM N^+-glucuronide under the same incubation conditions. This glucuronidation unique to humans is similar to findings by Kassahun et al. [43] on olanzapine *N*-glucuronidation that was studied in CD-1 mice, Fischer 344 rats, beagle dogs, and rhesus monkeys. 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 [42]. In the rat, the reason for lack of *N*-glucuronidating activity is suggested to result from a defect 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 highly species-dependent and may be an important elimination pathway for many tertiary amines in humans.

TAM N^+-glucuronide showed affinities very similar to TAM itself for human ERα and ERβ as a competitor to endogenous β-estradiol, suggesting that the formation of TAM N^+-glucuronide may not be only for a deactivation pathway. 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 [45]. However, no information is available on *N*-glucuronidation of 4-HO-TAM. Our preliminary results indicated that 4-HO-TAM also received *N*-glucuronidation by human liver microsomes. These data will be published elsewhere.

TAM is known as a mixed antiestrogen in which the biocharacter (agonist versus antagonist activity) varies among different tissues, cells, and promoters [46]. In view of the structure–binding activity relationship, it is reported that antiestrogens, such as 4-HO-TAM and raloxifene, require an alkylaminoethoxy side chain to block estrogen action [47]. However, the *N*,*N*-dimethyl-alkyl side chain of TAM is not essential for binding to ERs because *N*-demethylation or cleavage of the side chain from TAM reduces but not abolishes ER binding affinity [6]. The crystal structures of 4-HO-TAM:ER and raloxifene:ER complexes demonstrated that the antiestrogenic side chain interacts with aspartate at position 351 in the ER [48]. 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 antiestogens [48]. Therefore, it is possible that the *N*-glucuronidation of TAM might modulate the antiestrogenic activity of TAM. Further study will be required to determine whether TAM N^+-glucuronide acts as an antagonist or agonist for ER transcriptional activity. Furthermore, it should be determined whether N^+-glucuronide has biological significance in TAM therapy.

Detection and identification of TAM N^+-glucuronide from biological fluids of patients administered TAM are now in progress in our laboratory.

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References

[1] Jordan VC. Studies on the estrogen receptor in breast cancer-20 years as a target for the treatment and prevention of cancer. Breast Cancer Res Treat 1995;36:267–85.

[2] White INH. Tamoxifen: is it safe? Comparison of activation and detoxication mechanisms in rodents and in humans. Curr Drug Metab 2003;4:223–39.

[3] Ikeda K, Arao Y, Otsuka H, Nomoto S, Horiguchi H, Kato S, et al. Terpenoids found in the umbelliferae family act as agonists/antagonists for ERα and ERβ: differential transcription activity between ferutinine-liganded ERα and ERβ. Biochem Biophys Res Commun 2002;291:354–60.

[4] Bain RR, Jordan VC. Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy. Biochem Pharmacol 1983;32:373–5.

[5] Kemp JV, Adam HK, Wakeling AE, Slater R. Identification and biological activity of tamoxifen metabolites in human serum. Biochem Pharmacol 1983;32:2045–52.

[6] Lien EA, Solheim E, Kvinnsland S, Ueland PM. Identification of 4-hydroxy-*N*-desmethyltamoxifen as a metabolite of tamoxifen in human bile. Cancer Res 1988;48:2304–8.

[7] Poon GK, Chui YC, McCague R, Lønning PE, Feng R, Rowlands MG, et al. Analysis of phase I and phase II metabolites of tamoxifen in breast cancer patients. Drug Metab Dispos 1993;21:1119–24.

[8] Katzenellenbogen BS, Norman MJ, Eckert RL, Peltz SW, Mangel WF. Bioactivities, estrogen receptor interactions, and plasminogen activator-inducing activities of tamoxifen and hydroxy-tamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res 1984;44:112–9.

[9] Williams ML, Lennard MS, Martin IJ, Tucker GT. Interindividual variation in the isomerization of 4-hydroxytamoxifen by human liver microsomes: involvement of cytochromes P450. Carcinogenesis 1994;15:2733–8.

[10] Nishiyama T, Ogura K, Nakano H, Ohnuma T, Kaku T, Hiratsuka A, et al. Reverse geometrical selectivity in glucuronidation and sulfation of *cis*- and *trans*-4-hydroxytamoxifens by human liver UDP-glucuronosyltransferases and sulfotransferases. Biochem Pharmacol 2002;63:1817–30.

[11] Fromson JM, Pearson S, Bramah S. The metabolism of tamoxifen (I.C.I 46,474). II. In female patients. Xenobiotica 1973;3:711–4.

[12] Lien EA, Solheim E, Lea OA, Lundgren S, Kvinnsland S, Ueland PM. Distribution of 4-hydroxy-*N*-desmethyltamoxifen and other tamoxifen metabolites in human biological fluids during tamoxifen treatment. Cancer Res 1989;49:2175–83.

[13] Hawes EM. N^+-glucuronidation, a common pathway in human metabolism of drugs with a tertiary amine group. Drug Metab Dispos 1998;26:830–7.

[14] Luo H, Hawes EM, McKay G, Korchinski ED, Midha KK. N^+-glucuronidation of aliphatic tertiary amines, a general phenomenon in the metabolism of H_1-antihistamines in humans. Xenobiotica 1991;21:1281–8.

[15] Green MD, Tephly TR. Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. Drug Metab Dispos 1996;24:356–63.

[16] Mey U, Wachsmuth H, Breyer-Pfaff U. Conjugation of the enantiomers of ketotifen to four isomeric quaternary ammonium glucuronides in humans in vivo and in liver microsomes. Drug Metab Dispos 1999;27:1281–92.

[17] Breyer-Pfaff U, Mey U, Green MD, Tephly TR. Comparative *N*-glucuronidation kinetics of ketotifen and amitriptyline by expressed human UDP-glucuronosyltransferases and liver microsomes. Drug Metab Dispos 2000;28:869–72.

[18] Strassburg CP, Manns MP, Tukey RH. Differential down-regulation of the *UDP-glucuronosyltransferase 1A* locus is an early event in human liver and biliary cancer. Cancer Res 1997;57:2979–85.

[19] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

[20] Bansal SK, Gessner T. A unified method for the assay of uridine diphosphoglucuronyltransferase activities toward various aglycones using uridine diphospho[U-^{14}C]glucuronic acid. Anal Biochem 1980;109:321–9.

[21] Fisher MB, Campanale K, Ackermann BL, VandenBranden M, Wrighton SA. In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. Drug Metab Dispos 2000;28:560–6.

[22] Dehal SS, Gagne PV, Crespi CL, Patten CJ. Characterization of a probe substrate and an inhibitor of UDP-glucuronosyltransferase 1A4 activity in human liver microsomes and cDNA-expressed UGT-enzymes. Drug Metab Rev 2001;33:162 [abstract].

[23] Ritter JK, Chen F, Sheen YY, Tran HM, Kimura S, Yeatman MT, et al. A novel complex locus *UGT1* encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini. J Biol Chem 1992;267:3257–61.

[24] Ebner T, Remmel RP, Burchell B. Human bilirubin UDP-glucuronosyltransferase catalyzes the glucuronidation of ethinylestradiol. Mol Pharmacol 1993;43:649–54.

[25] Mojarrabi B, Butler R, Mackenzie PI. cDNA cloning and characterization of the human UDP glucuronosyltransferase, UGT1A3. Biochem Biophys Res Commun 1996;225:785–90.

[26] Harding D, Fournel-Gigleux S, Jackson MR, Burchell B. Cloning and substrate specificity of a human phenol UDP-glucuronosyltransferase expressed in COS-7 cells. Proc Natl Acad Sci USA 1988;85:8381–5.

[27] Wooster R, Sutherland L, Ebner T, Clarke D, Da Cruz e Silva O, Burchell B. Cloning and stable expression of a new member of the human liver phenol/bilirubin: UDP-glucuronosyltransferase cDNA family. Biochem J 1991;278:465–9.

[28] Fournel-Gigleux S, Jackson MR, Wooster R, Burchell B. Expression of a human liver cDNA encoding a UDP-glucuronosyltransferase catalysing the glucuronidation of hyodeoxycholic acid in cell culture. FEBS Lett 1989;243:119–22.

[29] Ritter JK, Chen F, Sheen YY, Lubet RA, Owens IS. Two human liver cDNAs encode UDP-glucuronosyltransferases with 2 log differences in activity toward parallel substrates including hyodeoxycholic acid and certain estrogen derivatives. Biochemistry 1992;31:3409–14.

[30] Jin C-J, Miners JO, Lilywhite KJ, Mackenzie PI. cDNA cloning and expression of two new members of the human liver UDP-glucuronosyltransferase 2B subfamily. Biochem Biophys Res Commun 1993;194:496–503.

[31] Ritter JK, Sheen YY, Owens IS. Cloning and expression of human liver UDP-glucuronosyltransferase in COS-1 cells. 3,4-Catechol estrogens and estriol as primary substrates. J Biol Chem 1990;265:7900–6.

[32] Jin C-J, Miners JO, Lilywhite KJ, Mackenzie PI. Complementary deoxyribonucleic acid cloning and expression of a human liver uridine diphosphate-glucuronosyltransferase glucuronidating carboxylic acid-containing drugs. J Pharmcol Exp Ther 1993;264:475–9.

[33] Coffman BL, Rios GR, King CD, Tephly TR. Human UGT2B7 catalyzes morphine glucuronidation. Drug Metab Dispos 1997;25:1–4.

[34] Beaulieu M, Lévesque É, Hum DW, Bélanger A. Isolation and characterization of a human orphan UDP-glucuronosyltransferase, UGT2B11. Biochem Biophys Res Commun 1998;248:44–50.

[35] Shepherd SR, Baird SJ, Hallinan T, Burchell B. An investigation of the transverse topology of bilirubin UDP-glucuronosyltransferase in rat hepatic endoplasmic reticulum. Biochem J 1989;259:617–20.

[36] Chen F, Ritter JK, Wang MG, McBride OW, Lubet RA, Owens IS. Characterization of a cloned human dihydrotestosterone/androstanediol UDP-glucuronosyltransferase and its comparison to other steroid isoforms. Biochemistry 1993;32:10648–57.
[37] Beaulieu M, Lévesque É, Hum DW, Bélanger A. Isolation and characterization of a novel cDNA encoding a human UDP-glucuronosyltransferase active on C_{19} steroids. J Biol Chem 1996;271:22855–62.
[38] Bélanger A, Hum DW, Beaulieu M, Lévesque É, Guillemette C, Tchernof A, et al. Characterization and regulation of UDP-glucuronosyltransferases in steroid target tissues. J Steroid Biochem Mol Biol 1998;65:301–10.
[39] Lévesque É, Turgeon D, Carrier J-S, Montminy V, Beaulieu M, Bélanger A. Isolation and characterization of the UGT2B28 cDNA encoding a novel human steroid conjugating UDP-glucuronosyltransferase. Biochemistry 2001;40:3869–81.
[40] Green MD, Bishop WP, Tephly TR. Expressed human UGT1.4 protein catalyzes the formation of quaternary ammonium-linked glucuronides. Drug Metab Dispos 1995;23:299–302.
[41] Green MD, King CD, Mojarrabi B, Mackenzie PI, Tephly TR. Glucuronidation of amines and xenobiotics catalyzed by expressed human UDP-glucuronosyltransferase 1A3. Drug Metab Dispos 1998; 26:507–12.
[42] Green MD, Tephly TR. Glucuronidation of amine substrates by purified and expressed UDP-glucuronosyltransferase proteins. Drug Metab Dispos 1998;26:860–7.
[43] Kassahun K, Mattiuz E, Franklin R, Gillespie T. Olanzapine 10-*N*-glucuronide. A tertiary *N*-glucuronide unique to humans. Drug Metab Dispos 1998;26:848–55.
[44] Bruck M, Li Q, Lamb JG, Tukey RH. Characterization of rabbit UDP-glucuronosyltransferase UGT1A7: tertiary amine glucuronidation is catalyzed by UGT1A7 and UGT1A4. Arch Biochem Biophys 1997; 348:357–64.
[45] McCague R, Parr IB, Leclercq G, Leung O-T, Jarman M. Metabolism of tamoxifen by isolated rat hepatocytes. Identification of the glucuronide of 4-hydroxytamoxifen. Biochem Pharmacol 1990;39:1459–65.
[46] Buckley MM, Goa KL. Tamoxifen. A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic use. Drugs 1989;37:451–90.
[47] Jordan VC. Biochemical pharmacology of antiestrogen action. Pharmacol Rev 1984;36:245–76.
[48] MacGregor Schafer J, Liu H, Bentrem DJ, Zapf JW, Jordan VC. Allosteric silencing of activating function 1 in the 4-hydroxytamoxifen estrogen receptor complex is induced by substituting glycine for aspartate at amino acid 351. Cancer Res 2000;60:5097–105.