Tranilast is an oral antiallergic agent widely used in Japan. Recently, in Western populations, hyperbilirubinemia induced by tranilast was suspected during clinical trials. Tranilast has been reported to be mainly metabolized to a glucuronide and a phase I metabolite, 4-demethyltranilast (N-3). In the present study, we investigated the in vitro metabolism of tranilast in human liver and jejunum microsomes and recombinant UDP-glucuronosyltransferases (UGTs). The glucuronidation of tranilast was clarified to be mainly catalyzed by UGT1A1 in human liver and intestine. The $K_m$ values of tranilast glucuronosyltransferase activity were 51.5, 50.6, and 38.0 μM in human liver microsomes, human jejunum microsomes, and recombinant UGT1A1, respectively. When the intrinsic clearance was calculated using the in vitro kinetic parameters, microsomal protein content, and weight of tissues, tranilast glucuronosyltransferase activity was strongly inhibited by bilirubin, a typical UGT1A1 substrate, and N-3, indicating that the phase I metabolite could affect the tranilast glucuronosyltransferase activity. In the case of N-3 formation, the $K_m$ and $V_{\text{max}}$ values were 37.1 μM and 27.6 pmol/min/mg protein in human liver microsomes. The bilirubin glucuronosyltransferase activity was strongly inhibited by both tranilast and N-3, suggesting that tranilast-induced hyperbilirubinemia would be responsible for the inhibition by tranilast and N-3 of the bilirubin glucuronosyltransferase activity, as would the UGT1A1 genotype.

Tranilast (N-(3’4’-demethoxycinnamoyl)-anthranilic acid) is an oral antiallergic agent developed by Kissei Pharmaceutical Co. Ltd. (Nagano, Japan) and widely used in Japan for bronchial asthma, allergic rhinitis, atopic dermatitis, keloid, and hypertrophic scar. The mechanism of its efficacy is to inhibit chemical mediators from mast cells (Azuma et al., 1976) and the accumulation of collagen in granulation tissue (Isaji et al., 1987). Recently, a clinical trial regarding the prevention of restenosis after percutaneous transluminal coronary revascularization was performed in Western populations (Holmes et al., 2000). During that trial, it was found that hyperbilirubinemia might be induced by tranilast and the risk of hyperbilirubinemia was increased in individuals with Gilbert’s syndrome (Danoff et al., 2004).

Major metabolic pathways of tranilast have been shown to be glucuronidation, 4-demethylation (N-3), and sulfation of N-3 in the data sheet of tranilast provided by Kissei Pharmaceutical (Fig. 1). Tranilast, N-3, and N-3 sulfate were reported to be detected in human urine (Slobodzian et al., 1985). Since the urine sample was hydrolyzed by glucuronidase and/or base in that article, the formation of glucuronide could be speculated by a comparison of the chromatograms before and after hydrolysis. The major metabolites of tranilast in urine in human were tranilast glucuronide and N-3 sulfate and their recoveries were almost the same (unpublished report from Kissei Pharmaceutical). Although phase I metabolism of tranilast was shown to be mainly catalyzed by CYP2C9 in humans (unpublished data from Kissei Pharmaceutical), tranilast metabolism in glucuronidation still remains uncertain.

In the case of many drugs, since a parent drug and/or its phase I metabolite could be conjugated with glucuronic acid, the role of UDP-glucuronosyltransferase (UGT) has recently received attention. The major metabolic pathway of a drug is not always catalyzed by cytochrome P450. In the case of tranilast, N-3 formation was catalyzed by CYP2C9, and the possibility of drug interaction with warfarin was described in the data sheet of tranilast. However, the kinetics of tranilast glucuronidation is unknown. The purpose of the present study was to clarify the tranilast metabolism involving glucuronidation. In addition, to investigate the mechanism of tranilast-induced hyperbilirubinemia, the inhibitory effects of tranilast and N-3 on bilirubin glucuronosyltransferase activity were demonstrated.

Materials and Methods

Materials. Tranilast, 4-demethyltranilast, and 3-demethyltranilast (N-4) were kindly supplied by Kissei Pharmaceutical. UDP-glucuronic acid (UDP-GA), alamethicin, β-estradiol, emodin, and β-glucuronidase from Helix pomatia (type H-2) were purchased from Sigma-Aldrich (St. Louis, MO). Biliarubin, 4-nitrophenol, and imipramine hydrochloride were obtained from Wako.
Pure Chemicals (Osaka, Japan). Propofol was kindly provided by AstraZeneca (London, UK). Nicotinamide adenine dinucleotide phosphate (oxidized form, NADP\(^{+}\)) and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). 7-Hydroxycoumarin was obtained from Invitrogen (Carlsbad, CA). Pooled human liver microsomes and microsomes from 22 individual human livers were purchased from BD Gentest (Woburn, MA). The glucuronosyltransferase activity of \(\beta\)-estradiol in these human individual liver microsomes was provided as the typical activity for UGT1A1 by the manufacturer. The human jejunum microsomes (HJM0040) were purchased from KAC (Kyoto, Japan). Recombinant human UGT1A8, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 expressed in baculovirus-infected insect cells were also obtained from BD Gentest. All other chemicals and solvents were of analytical or the highest grade commercially available.

**Tranilast Glucuronidation Assay.** A typical incubation mixture (total volume, 0.2 ml) contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl\(_2\), 50 \(\mu\)g of alamethicin/mg microsomal protein, 5 mM UDP-GA, 0.5 mg/ml human liver microsomes, and tranilast. The reaction was initiated by the addition of UDP-GA and the mixture was then incubated for 60 min at 37°C. The reaction was terminated by boiling for 5 min. After removal of the protein by centrifugation at 9000g for 5 min, an 80-\(\mu\)l portion of the sample was subjected to high-performance liquid chromatography (HPLC) with a NovaPack Phenyl 4-\(\mu\)m analytical column (3.9 \(\times\) 150 mm; Waters, Milford, MA). The product formation was measured as described previously (Slobodzian et al., 1985) with slight modifications. The mobile phase was methanol/50 mM sodium dihydrogen phosphate (pH 5.3), 40:60 (v/v) and the flow rate was 1.0 ml/min. The eluent was monitored at 335 nm with a noise-base clean Uni-3 (Union, Gunma, Japan). The retention times of tranilast glucuronide, tranilast, and 7-hydroxycoumarin (internal standard, IS) were 4.4, 10.0, and 3.6 min, respectively (Fig. 2). None of these chromatograms showed any interfering peaks with tranilast glucuronide. For the quantification of tranilast glucuronide, the eluate of the HPLC from the incubation mixture with human liver microsomes, including tranilast glucuronide, was collected with reference to the retention time. A part of the eluate was incubated with 1000 U/ml \(\beta\)-glucuronidase at 37°C for 24 h. The hydrolyzed tranilast glucuronide was quantified as tranilast by HPLC.

The hydrolyzed tranilast glucuronide was quantified as tranilast by HPLC. Once we determined the peak area per known content of tranilast glucuronide, the ratio was applied to the calculation of the tranilast glucuronide formed in the incubation mixtures.

**Kinetic Analyses.** The kinetic studies were performed using human liver microsomes, human jejunum microsomes, and recombinant human UGT1A1.
expressed in baculovirus-infected insect cells. When determining the kinetic parameters, the tranilast concentration ranged from 5 μM to 2 mM. The kinetic parameters were estimated from the fitted curves using a computer program, KaleidaGraph (Synergy Software, Reading, PA), designed for nonlinear regression analysis.

Correlation Analyses. The correlations between the tranilast glucuronosyltransferase activity and the other glucuronosyltransferase activities were determined by Pearson’s product moment method. A p value of less than 0.05 was considered statistically significant.

Inhibition Analysis of Tranilast Glucuronosyltransferase Activity in Human Liver and Jejunum Microsomes. As described by Watanabe et al. (2002), bilirubin (UGT1A1), α-estradiol (UGT1A1 and UGT1A9), 4-nitropheno- nol (UGT1A6 and UGT1A9), imipramine (UGT1A3 and UGT1A4), emodin (UGT1A8 and UGT1A10), and propofol (UGT1A9) are typical substrates for each UGT isoform. These six substrates were investigated for their inhibitory effects on the tranilast glucuronosyltransferase activity. For the determination of the IC50 values, the concentration of tranilast was set at 100 μM. The final concentration of the organic solvents in the reaction mixture was <2% (v/v).

The tranilast glucuronosyltransferase activities in pooled human liver microsomes and human jejunum microsomes (HJM0040) at 100 μM tranilast were determined as described above.

Inhibition of N-3 and N-4 on Tranilast Glucuronosyltransferase Activity in Human Liver and Jejunum Microsomes. The inhibition of N-3, a phase I metabolite of tranilast, and N-4, a structural isomer of N-3, on the tranilast glucuronosyltransferase activity was also investigated in pooled human liver microsomes and individual human jejunum microsomes. For the determination of the IC50 values, the concentration of tranilast was set at 100 μM. For the determination of the K m values in pooled human liver microsomes, the concentrations of tranilast, N-3, and N-4 ranged from 10 to 160 μM, 0 to 150 μM, and 0 to 30 μM, respectively. The K m values were estimated from the fitted curve using a computer program (K cat; BioMetallcits, Princeton, NJ) designed for nonlinear regression analysis.

Tranilast 4-Demethylation Assay. A typical incubation mixture (total volume, 0.2 ml) contained 100 mM Tris-HCl buffer (pH 7.4), 0.2 mg/ml human liver microsomes, an NADPH-generating system (0.5 mM NADP +, 5 mM glucose 6-phosphate, 5 mM MgCl 2, and 1 μM glucose-6-phosphate dehydrogenase), and tranilast. The reaction was initiated by the addition of the NADPH-generating system and was then incubated for 30 min at 37°C. The reaction was terminated by adding 100 μl of ice-cold methanol. 7-Hydroxy- coumarin was added as an IS. After removal of the protein by centrifugation at 9000g for 5 min, an 80-μl portion of the sample was subjected to HPLC. The product formation was measured using the same method for tranilast glucuronide except the mobile phase. The mobile phase was methanol/50 mM sodium dihydrogen phosphate (pH 5.3), 33:67 (v/v). The retention times of N-3, tranilast, and 7-hydroxycoumarin (IS) were 9.8, 21.4, and 4.7 min, respectively. None of these chromatograms showed any interfering peaks with N-4 (data not shown).

The kinetic studies were performed using human liver microsomes. In determining the kinetic parameters, the tranilast concentration ranged from 2 to 500 μM. Kinetic parameters were estimated from the fitted curves using the computer program KaleidaGraph (Synergy Software) designed for nonlinear regression analysis.

Inhibition of Tranilast, N-3, and N-4 on Bilirubin Glucuronosyltrans- ferase Activity in Human Liver Microsomes. A typical incubation mixture (total volume, 0.2 ml) contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl 2, 50 μg of alamethicin/mg microsomal protein, 2 mM UDP-GA, 0.5 mg/ml human liver microsomes, 10 μM bilirubin, and tranilast (N-3 or N-4). The reaction was initiated by the addition of UDP-GA and was then incubated for 30 min at 37°C. The reaction was terminated by adding 100 μl of ice-cold methanol. After removal of the protein by centrifugation at 9000g for 5 min, a 50-μl portion of the sample was subjected to HPLC with a Develosil C30 5-μm analytical column (4.6 × 150 mm; Nomura Chemical, Aichi, Japan). The product formation was measured as described previously (Lacquita et al., 2001) with slight modifications. The mobile phase was 55% methanol/50 mM potassium dihydrogen phosphate and the flow rate was 1.0 ml/min. The eluent was monitored at 450 nm with a noise-base clean Uni-3 (Union). The final concentration of the organic solvents in the reaction mixture was <2% (v/v).

Results

Kinetics of Tranilast Glucuronosyltransferase Activity in Hu- man Liver or Jejunum Microsomes. Kinetic analyses of tranilast glucuronidation in human liver or jejunum microsomes were performed. As shown in Fig. 3, A and B, the kinetics of 5 to 500 μM tranilast fitted to the Michaelis-Menten equation (5 to 500 μM, closed circles). The tranilast glucuronosyltransferase activity was determined as described under Materials and Methods. Each data point represents the mean of duplicate determinations.

Inhibition Analysis of Tranilast Glucuronosyltransferase Activity from 22 Human Livers and Correlation Analyses. The tranilast glucuronosyltransferase activities in microsomes from 22 human livers were determined at 40 μM tranilast (Fig. 5, top). The tranilast glucuronosyltransferase activity ranged from 1.9 pmol/min/mg protein in HG93 to 18.3 pmol/min/mg protein in HH31. The interindividual variability in the tranilast glucuronosyltransferase activity was 9.5-fold. Correlation analyses were performed between the tranilast glucuronosyltransferase activity and bilirubin (UGT1A1), α-estradiol (UGT1A1), etoposide (UGT1A1), trifluoperazine (UGT1A4), propofol (UGT1A9), or morphine glucuronosyltransferase activities (UGT2B7) provided by the manufacturer (Table 1).
The etoposide glucuronosyltransferase activities (UGT1A1) were measured in our laboratory according to the method of Watanabe et al. (2002). Since we could not obtain those activities in all individual liver microsomes, correlation analyses among bilirubin, etoposide, and morphine glucuronosyltransferase activities were performed using 11 of 22 liver microsomes. The tranilast glucuronosyltransferase activities in individual human liver microsomes were significantly correlated with the trifluoperazine (r = 0.956, p < 0.0001), bilirubin (r = 0.937, p < 0.0001), and propofol (r = 0.449, p < 0.05) glucuronosyltransferase activities. The tranilast glucuronosyltransferase activities did not correlate with the trifluoperazine (r = 0.179) and morphine (r = 0.257) glucuronosyltransferase activities.

**Inhibition Analyses of Glucuronosyltransferase Activity in Human Liver or Jejunum Microsomes.** The inhibitory effects of bilirubin, β-estradiol, 4-nitrophenol, imipramine, emodin, and propofol on the tranilast glucuronosyltransferase activity in human liver and jejunum microsomes were determined. As shown in Fig. 6A, the tranilast glucuronosyltransferase activity in pooled human liver microsomes was inhibited by bilirubin (IC_{50} = 123.9 μM). As shown in Fig. 6B, the activity in human jejunum microsomes was strongly inhibited by bilirubin (IC_{50} = 81.1 μM) and β-estradiol (IC_{50} = 75.3 μM).

**Inhibition of N-3 and N-4 on Tranilast Glucuronosyltransferase Activity in Human Liver and Jejunum Microsomes.** The inhibitory effects of N-3 and N-4 on the tranilast glucuronosyltransferase activity in human liver and jejunum microsomes were determined. The tranilast glucuronosyltransferase activities in human liver and jejunum microsomes were strongly inhibited by both N-3 and N-4 (Fig. 6). The IC_{50} values of N-3 and N-4 were 141.7 and 81.3 μM, respectively, in liver microsomes and 82.8 and 45.9 μM, respectively, in jejunum microsomes. The inhibition pattern of N-3 was competitive. The K_{m} value of N-3 for the tranilast glucuronosyltransferase activity in human liver microsomes was 52.8 μM (Fig. 7). On the other hand, the inhibition pattern of N-4 was mixed. The K_{m} and V_{max} values of N-4 for the tranilast glucuronosyltransferase activity in human liver microsomes were 42.6 and 181.1 μM, respectively (Fig. 7).

**Kinetics of Tranilast 4-Demethylase Activity in Human Liver Microsomes.** The kinetics of tranilast 4-demethylation (N-3 formation) in pooled human liver microsomes at 2 to 500 μM tranilast fitted to the Michaelis-Menten kinetics. The K_{m} and V_{max} values were 37.1 ± 3.1 μM and 27.6 ± 0.7 pmol/min/mg protein, respectively.

**Interindividual Variability of Tranilast 4-Demethylase Activity from 22 Human Livers and Correlation Analyses.** The tranilast 4-demethylase activities in microsomes from 22 human livers were determined at 40 μM tranilast. The interindividual variability in the tranilast glucuronosyltransferase activity was at most 16.4-fold (Fig. 5, bottom). The tranilast 4-demethylase activity ranged from 4.5 pmol/min/mg protein in HG32 to 73.1 pmol/min/mg protein in HG30. Correlation analyses were performed between the tranilast 4-demethylase activity and phenacetin O-deethylation activity (CYP1A2), coumarin 7-hydroxylation activity (CYP2A6), S-mephenytoin N-demethylase activity (CYP2B6), paclitaxel 60-hydroxylation activity (CYP2C8), diclofenac 4’-hydroxylation activity (CYP2C9), S-mephenytoin 4’-hydroxylation activity (CYP2C19), bufuralol 1’-hydroxylation activity (CYP2D6), chlorzoxazone 6-hydroxylation activity (CYP2E1), testosterone 6β-hydroxylation activity (CYP3A4), and lauric acid 12-
hydroxylase activity (CYP4A) provided by the manufacturer. The tranilast 4-demethylase activities in the 22 human liver microsomes were significantly correlated with the diclofenac 4'-hydroxylation activities ($r = 0.825$, $p < 0.0001$) and the paclitaxel 6a-hydroxylase activities ($r = 0.576$, $p < 0.01$).

**Inhibition of Tranilast, N-3, and N-4 on Bilirubin Glucuronosyltransferase Activity in Human Liver Microsomes.** The inhibitory effects of tranilast, N-3, and N-4 on the bilirubin glucuronosyltransferase activity in human liver microsomes were determined. The bilirubin glucuronosyltransferase activity was strongly inhibited by tranilast, N-3, and N-4. The $IC_{50}$ values of tranilast, N-3, and N-4 were 28.7, 76.9, and 62.0 μM, respectively.

**Discussion**

Although tranilast has been prescribed for many decades in Japan, its metabolism has not been investigated completely. In human, the major metabolites have been reported to be tranilast glucuronide and N-3 sulfate (unpublished data, Kissei Pharmaceutical). In human urine, other metabolites such as N-2 [2-(4'-hydroxy-3'-methoxy-styryl)-3,1-benzoxazin-4-one], N-6 (1-benzoxazin-4-one), and N-3 glucuronide (unpublished data, Kissei Pharmaceutical) were slightly detected. In the present study, it was clarified that tranilast glucuronidation was mainly catalyzed by UGT1A1 in human. UGT1A3, UGT1A8, and UGT1A10 were partly responsible for the tranilast glucuronidation. The tranilast glucuronosyltransferase activity could be detected in both human liver and jejunum microsomes. UGT1A1 is one of the major isoforms of UGTs in the liver and is also expressed in the intestine. UGT1A8 and UGT1A10 could be responsible for the extrahepatic metabolism of tranilast because these isoforms are expressed in the intestine but not in liver. However, the tranilast glucuronosyltransferase activity in human jejunum microsomes was inhibited by bilirubin and β-estradiol but not by emodin (Fig. 6B), suggesting that the tranilast glucuronosyltransferase activity was mainly catalyzed by UGT1A1 in intestine as in the liver.

The tranilast glucuronosyltransferase activity was reduced at high substrate concentrations (>500 μM) in human liver and jejenum microsomes and recombinant UGT1A1. The reason for this phenomenon was unclear, but substrate and/or metabolite inhibition may be involved. Further study is needed to clarify the mechanism of the inhibition. The maximum serum concentration of tranilast in humans has been reported to be 37.0 μM after single oral administration at the therapeutic dose of 100 mg (Slobodzian et al., 1985). As reported by Kissei Pharmaceutical, the maximum serum concentration of tranilast was 67.8 μM after taking 2.5 mg/kg tranilast three times per day for 5 days. In clinical practice, the concentration of tranilast is unlikely to reach 500 μM. Therefore, the kinetic parameters fitted to the Michaelis-Menten equation with <500 μM tranilast seems to be reasonable.

The in vitro intrinsic clearance ($CL_{\text{int}}$) is calculated using the following equation (Obach et al., 1997; Soars et al., 2002): $CL_{\text{int}} = \frac{[V_{\text{max}}/K_{\text{m}}] \times [\text{microsomal protein/tissue (mg/g)}] \times [\text{tissue/body weight (g/kg)}]}{[\text{microsomal protein/g of liver and 20 g of liver/kg of body weight. The } CL_{\text{int}} \text{ in liver of tranilast was calculated to be } 181.7 \mu l/min/kg. They also reported that there are 3 mg of microsomal protein/g of liver and 20 g of liver/kg of body weight (Soars et al., 2002). The } CL_{\text{int}} \text{ in intestine was estimated to be } 76.3 \mu l/min/kg. The glucuronosyltransferase activity has been reported to differ according to regions of the intestine in humans (Strassburg et al., 2000). The UGT1A1 activity in humans was higher in upper intestine than lower intestine (Basu et al., 2004). Although the UGT activity in the intestine may differ from that in the liver, the tranilast glucuronosyltransferase activity in the intestine might be approximately 40% of that in the liver.
UGT1A1 has shown polymorphic metabolism. Particularly, the relationship between the toxicity of irinotecan hydrochloride and the UGT1A1 genotype has been extensively studied (Ando et al., 2005). As well as irinotecan hydrochloride, UGT1A1 genetic polymorphism would affect the tranilast pharmacokinetics.

Another metabolic pathway of tranilast is N-3 formation catalyzed by CYP2C9 (unpublished data, Kissei Pharmaceutical). We first analyzed the kinetics of tranilast 4-demethylase activity in human liver microsomes. The $K_m$ value of N-3 formation in human liver microsomes was $37.1 \pm 3.1 \mu M$, which was similar to that of the tranilast glucuronosyltransferase activity. There was large interindividual variability (16-fold) in the N-3 formation. In CYP2C9, several polymorphic alleles have been reported to have decreased enzyme activity in vitro and in vivo (Takahashi et al., 1998; Blaisdell et al., 2004). The frequency of poor metabolizing alleles such as CYP2C9*2 and CYP2C9*3 was different between ethnic groups (Sullivan-Klose et al., 1996; Blaisdell et al., 2004). Therefore, the genetic polymorphisms of CYP2C9 and UGT1A1 would play important roles in the pharmacokinetics of tranilast.

In the present study, N-3 was demonstrated to inhibit tranilast glucuronosyltransferase activity. Although the concentration of N-3 in the liver is unknown, N-3 could affect tranilast glucuronosyltransferase activity, leading to altered pharmacokinetics of tranilast. N-3 is further metabolized to be N-3 glucuronide and could be slightly detected in human urine (unpublished data, Kissei Pharmaceutical).

Since the inhibition pattern of N-3 on tranilast glucuronosyltransferase activity was competitive, the glucuronidation of N-3 might be catalyzed by UGT1A1. N-4, a structural isomer of N-3, also inhibited tranilast glucuronosyltransferase activity. However, there are no reports that N-4 could be detected in humans. Because glucuronidation of a drug may be inhibited by its cytochrome P450 metabolites, the inhibition by metabolites should be kept in mind when estimating the pharmacokinetics.

Recently, the PRESTO (prevention of restenosis with tranilast and its outcomes) study was performed in Western populations because tranilast may have a benefit in preventing restenosis after percutaneous transluminal coronary revascularization (Holmes et al., 2000). During the phase III clinical trial, an increase in serum unconjugated bilirubin after percutaneous transluminal coronary revascularization (Holmes et al., 2000). During the phase III clinical trial, an increase in serum unconjugated bilirubin after percutaneous transluminal coronary revascularization (Holmes et al., 2000).

In conclusion, it was clarified that the tranilast glucuronosyltransferase activity was mainly catalyzed by UGT1A1 in human liver and intestine. N-3, a phase I metabolite of tranilast, inhibited the tranilast glucuronosyltransferase activity, suggesting that the inhibition by a phase I metabolite may be noteworthy when estimating the glucuronidation of a drug. The inhibition by tranilast and N-3 of the bilirubin glucuronosyltransferase activity may be partly responsible for tranilast-induced hyperbilirubinemia. We should keep in mind that UGT1A1 substrates may inhibit the bilirubin glucuronosyltransferase activity leading to hyperbilirubinemia, and that phase I metabolites can affect the glucuronidation of its parent drug.

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References


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