Dabigatran Acylglucuronide, the Major Human Metabolite of Dabigatran: In Vitro Formation, Stability, and Pharmacological Activity

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ABSTRACT:
Glucuronidation of the carboxylate moiety is the major human metabolic pathway of dabigatran (β-alanine, N-[[2-[[4-(aminomethyl)phenyl]amino]methyl]-1-methyl-1H-benimidazol-5-yl][carbonyl]-N-2-pyridinyl). It results in the formation of the 1-O-acylglucuronide. Four isomeric acylglucuronides of dabigatran were isolated and purified from urine of dosed rhesus monkeys. NMR analysis confirmed the structures of the four metabolites as the 1-O-acylglucuronide (β anomer) and the 2-O-, 3-O-, and 4-O-acylglucuronides (α and β anomers). Experiments with the purified 1-O-acylglucuronide and its isomeric rearrangement products revealed equipotent prolongation of the activated partial thromboplastin time compared with dabigatran. The 1-O-acylglucuronide, in addition to minor hydrolysis to the aglycon, underwent nonenzymatic acyl migration in aqueous solution, resulting in the formation of the 2-O-, 3-O-, and 4-O-acylglucuronides with an apparent half-life of 1 h (37°C, pH 7.4). The glucuronidation of dabigatran was catalyzed by human hepatic and intestinal microsomes with \( K_m \) values in the range of 180 to 255 and 411 to 759 \( \mu \)M, respectively. Three UDP-glucuronosyltransferases (UGTs), namely, UGT1A9, UGT2B7, and UGT2B15, exhibited glucuronidation of dabigatran. Based on a comparison of the in vitro intrinsic clearances, UGT2B15 was considered the major contributor to the glucuronidation of dabigatran. The major contribution of UGT2B15 and the minor contribution of at least two more UGT enzymes together with the lack of potent inhibition of dabigatran glucuronidation by several potential UGT inhibitors indicate a low risk of interaction by comedication on dabigatran glucuronidation in the clinic.

Introduction
For a large number of drugs, as well as many xenobiotics and endogenous compounds, glucuronidation constitutes a major metabolic pathway. Conjugation with β-D-glucuronic acid can substantially alter the structure and structure-related properties of a compound and thus may also modulate changes in the distribution and elimination of its substrates. In rare cases, glucuronidation results in the formation of pharmacologically active molecules.

Dabigatran (β-alanine, N-[[2-[[4-(aminomethyl)phenyl]amino]methyl]-1-methyl-1H-benimidazol-5-yl][carbonyl]-N-2-pyridinyl) is a reversible, competitive, direct thrombin inhibitor that is currently approved in Europe for the prevention of deep vein thrombosis in patients undergoing elective hip or knee replacement (Eriksson et al., 2005). Dabigatran is a highly polar (logD at pH 7.4 of −0.6), zwitterionic compound that is not suitable for oral dosing. Instead, the double prodrug dabigatran etexilate (β-alanine, N-[[2-[[4-[[hexyloxy]carbonyl]aminomethyl]phenyl]amino]methyl][1-methyl-1H-benimidazol-5-yl][carbonyl]-N-2-pyridinyl]-ethyl ester, methanesulfonate) is used for oral drug therapy. The double prodrug is rapidly converted to dabigatran by esterase-catalyzed hydrolysis after dosing in humans (Fig. 1). Dabigatran, being a polar compound, exhibits only a little oxidative metabolism. Instead, the acylglucuronide of the carboxylate functional group is formed, and this is the major metabolite in humans (Blech et al., 2008). After oral administration, approximately 20% of dabigatran is conjugated by glucurono-syltransferases to the pharmacologically active glucuronide conjugates (Stangier, 2008).

Acylglucuronides are ester-structured compounds that are chemically unstable in aqueous solution because of the susceptibility of the acyl group to nucleophilic attack and undergo both spontaneous hydrolysis and intramolecular acyl migration. Isomeric acylglucuronides, being reactive esters, have been shown to react with proteins to form covalent adducts. Covalent binding is a general phenomenon for labile acylglucuronides, and this protein binding may be responsible for adverse reactions because these chemically modified proteins may be immunogenic in vivo (Gillette, 1974). The formation of isomeric acylglucuronides via acyl migration is a prerequisite for covalent binding to proteins by the imine mechanism, in which the aldehyde group of the ring-open tautomer of the glucuronic acid moiety condenses with primary amino functional groups of proteins. Data on the reaction rates of degradation of 1-O-acylglucuronides can

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; ADME, absorption, distribution, metabolism, elimination; HPLC, high-performance liquid chromatography; COSY, correlation spectroscopy; PPP, platelet-poor plasma; aPTT, activated partial thromboplastin time; MES, 4-morpholineethanesulfonic acid; UDPGA, UDP-glucuronic acid; AUC, area under the plasma concentration-time curve.
serve as a measure for the potential of 1-O-acylglucuronides to covalently bind to proteins, as a correlation has been shown between the rate of degradation of 1-O-acylglucuronides (hydrolysis and acyl migration) in aqueous buffer and the extent of in vitro covalent binding to proteins (Benet et al., 1993; Bischer et al., 1995; Bailey and Dickinson, 1996). Therefore, data on the chemical stability of 1-O-acylglucuronide of dabigatran are considered to provide relevant information regarding the safety of dabigatran.

In this article, we describe how the isomeric acylglucuronides were isolated and purified from the urine of rhesus monkeys dosed with dabigatran etexilate after chemical syntheses of the 1-O-acylglucuronide of dabigatran proved to be not feasible. The isolated isomeric acylglucuronides were tested for anticoagulant activity. Formation of the acylglucuronides was characterized using in vitro systems and the UDP-glucuronosyltransferases (UGTs) that are involved in the formation of dabigatran acylglucuronide were elucidated.

Materials and Methods

Test Compounds, Chemicals and Reagents, and Other Materials.

Dabigatran and dabigatran etexilate were synthesized and analyzed at Boehringer Ingelheim GmbH and Co. KG (Biberach, Germany). Other chemicals were of analytical grade or higher purity and were obtained from commercial suppliers.

Human liver microsomes of pooled liver tissue (pool 1, pool of five male and five female white donors) were prepared by homogenization of the tissue samples with ice-cold 0.1 M phosphate buffer, pH 7.4, containing 1.15% potassium chloride followed by differential centrifugation. The 100,000 × g pellet was resuspended in 20 mM Tris buffer, pH 7.6 (pH at ambient temperature, pH 7.4 at 37°C), containing 0.25 M sucrose and 5.4 mM EDTA. The suspension was divided into aliquots, shock frozen in liquid nitrogen, and stored at −80°C until used for experiments. Pooled human liver microsomes (pool 2, pool of 50 donors, both genders) and samples of individual human liver microsomes were purchased from commercial sources (XenoTech, LLC, Lebanon, KS). Individual human intestinal microsomes (ileum and jejunum tissue pool of 50 donors, both genders) and samples of individual human liver microsomes were purchased from commercial sources (XenoTech, LLC, Lebanon, KS). Human liver microsomes of pooled liver tissue (pool 1, pool of five male and five female white donors) were prepared by homogenization of the tissue samples with ice-cold 0.1 M phosphate buffer, pH 7.4, containing 1.15% potassium chloride followed by differential centrifugation. The 100,000 × g pellet was resuspended in 20 mM Tris buffer, pH 7.6 (pH at ambient temperature, pH 7.4 at 37°C), containing 0.25 M sucrose and 5.4 mM EDTA. The suspension was divided into aliquots, shock frozen in liquid nitrogen, and stored at −80°C until used for experiments. Pooled human liver microsomes (pool 2, pool of 50 donors, both genders) and samples of individual human liver microsomes were purchased from commercial sources (XenoTech, LLC, Lebanon, KS). Individual human intestinal microsomes (ileum and jejunum tissue pool of 50 donors, both genders) and samples of individual human liver microsomes were purchased from commercial sources (XenoTech, LLC, Lebanon, KS).

Gentest Supersomes expressing human UGT isoforms were purchased from BD Gentest (Woburn, MA). These included microsomes from baculovirus-infected insect cells expressing UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 and control microsomes prepared from cells without the human liver UGT cDNA insert. All microsome samples were stored at −80°C until used for experiments.

Urine Samples of Rhesus Monkeys Containing Dabigatran Metabolites.

Urine samples were collected from male and female rhesus monkeys after repeated dosing with 500 mg/kg dabigatran etexilate methanesulfonate during an oral (gavage) maximum tolerated dose study that was part of the preclinical safety testing. The animals were kept in metabolic cages allowing complete collection of urine and feces. Urine fractions 0 to 24 h after dabigatran etexilate dosing were used for metabolite isolation. For stabilization of the labile acylglucuronides, urine was collected into cooled (crushed ice) vessels containing 50 ml of 0.1 M hydrochloric acid. After the collection of urine was completed, urine samples were pooled and immediately frozen at −20°C.

A small quantity of 14C-labeled dabigatran 1-O-acylglucuronide was obtained in the course of the ADME study in rhesus monkeys after intravenous dosing of 0.3 mg/kg [14C]dabigatran. 14C-labeled dabigatran 1-O-acylglucuronide was isolated from collected urine fractions and purified by XAD-2 extraction and HPLC as described below for nonlabeled material.

Isolation and Chromatographic Purification of Acylglucuronides.

Fifty microliters of the acidified urine samples (pH adjusted to −3.5) were extracted by solid-phase extraction using ServaChrom XAD type 2 resin in a glass column (20 × 1.5 cm, 10 × 1.5 cm resin bead dimensions). The column was washed with 150 ml of methanol and 300 ml of water. The urine samples were slowly applied onto the column. The column was then rinsed with 100 ml of water and was eluted with 50 ml of acetone/nitrite. The acetone/nitrite column eluent was evaporated under reduced pressure at 40°C until near dryness. Two microliters of water was added, and the aqueous solution was then divided in 200-μl aliquots and frozen at −20°C. Up to 100 μl of the aqueous XAD 2 extract was injected onto the HPLC column (Kromasil 100 C18, 5 μm; Vertex, Berlin, Germany). Metabolites were separated with a gradient of aqueous ammonium acetate (0.05 M, pH 4.8, mobile phase A) and methanol (mobile phase B) at a flow rate of 1.0 ml/min at 40°C (gradient: 100% A at 0 min, linear increase to 10% B at 15 min, plateau at this composition to 20 min, linear increase to 20% B at 30 min, plateau at this composition to 35 min, linear increase to 30% B at 40 min, and then 100% B for 2 min). Eluting peaks were detected by UV absorption at 292 nm. The HPLC column eluent fractions of four metabolite peaks consisting of putative acylglucuronides and the fraction containing the parent compound were collected into cooled (0°C) glass vials. The pH of collected metabolite fractions was tested and adjusted to moderately acidic conditions by addition of 25 μl of acetic acid. The organic solvent fraction of the metabolite containing column eluent was subsequently evaporated under reduced pressure and the water was removed by lyophilization. The dry metabolite samples were used for NMR analysis and incubation experiments in buffer for the evaluation of acyl migration.

NMR Analysis. 1H NMR and 1H,1H COSY spectra were recorded on a DRX 600 spectrometer (Bruker, Rheinstetten, Germany) using Bruker standard software and pulse programs. Tetramethylsilane was used as an internal standard. The solvent used was [2H₅]pyridine with a trace of trifluoroacetic acid as solvent according to the method of Kuo and Dulik (1995) to attain a downfield shift of the water signal.

In Vitro Stability of Dabigatran 1-O-Acylglucuronide in Aqueous Buffer.

The dry metabolite fraction that corresponded to the 1-O-acylglucuronide dabigatran was redissolved in a small volume (200 μl) of water, and 50 μl of this solution was pipetted into 450 μl of 0.1 M phosphate buffer. The pH of the resulting solution was adjusted to 7.4, and an aliquot of 50 μl was immediately removed (0 h). The resulting incubation mixtures contained approximately 13 μg/ml dabigatran 1-O-acylglucuronide (estimation based on the assumption of similar extinction coefficient for dabigatran 1-O-acetylglucuronide and parent compound). This solution was then incubated at 37°C. Sample aliquots of 50 μl were taken at time points 10, 20, and 40 min, and 1, 2, 4, 6, and 8 h. The samples were immediately mixed with 20 μl of 20% (v/v) acetic acid and were frozen at −80°C until used for HPLC analysis (described below). The area of each compound peak was measured at 292 nm. For calculations of the sum of the integrated peak, areas were set to 100% for each metabolite. The area of each compound peak was measured at 292 nm. For calculations of the sum of the integrated peak, areas were set to 100% for each metabolite.

Assessment of Anticoagulant Activity of Dabigatran Acylglucuronides.

Isolated dabigatran acylglucuronides were dissolved in 500 μl of water. The pH of the test solutions was adjusted to 5.5 by addition of aqueous acetic acid, and they were stored at 0°C. Reference solutions of dabigatran were also prepared. Venous blood from the antecubital vein was collected from three healthy donors. Blood samples were mixed with trisodium citrate to yield a final concentration of 10.6 mM citrate, pH 7.7 (1:10 dilution in whole blood) and centrifuged at 3500g for 15 min at 4°C to obtain platelet-poor plasma (PPP) that was stored immediately at −20°C. For some experiments, pooled
PFP of several donors was used. Coagulation assays were performed using aliquots of fresh frozen PPP in a four-channel CL-4 coagulometer (Behnk Elektronik, Norderstedt, Germany) or a Biomatik coagulometer (Desaga GmbH, Wiesloch, Germany). The test solutions (100-fold higher concentration than in the assay) were added to the PPP to yield the final assay concentrations. A volume of 50 μl of partial thromboplastin time reagent (Diagnostica Stago, Mannheim, Germany) was incubated with 50 μl of PPP from individual donors for 3 min at 37°C with continuous stirring, and coagulation was initiated with 50 μl of 25 mM calcium chloride solution. Assays with the Biomatik coagulometer were performed using 100-μl volumes of thromboplastin time reagent, pooled plasma, and calcium chloride solution. The time lag between the addition of calcium chloride solution and onset of clotting was determined as coagulation time. Duplicate measurements of aPTT from three individual donors or quadruplicate measurements using a pooled PPP sample were made. Dabigatran was tested in final concentrations ranging from 10 nM to 10 μM. The actual concentration of the 1-O-acylglucuronide in the test solutions was quantified (using a conversion factor for the extinction coefficient for the acylglucuronides of dabigatran; see Quantitative HPLC Analysis of Incubation Experiments) because the acylglucuronides were not available as chemically defined test articles. The anticoagulant activity of dabigatran 1-O-acylglucuronide was tested at concentrations ranging from 0.3 nM to 8.8 μM. The anticoagulant activity of the isomeric acylglucuronides was compared at a fixed concentration of 1.76 μM. Control samples that were treated identically to the coagulation assay samples but did not contain calcium chloride were used for the quantification of dabigatran. This procedure controlled for false-positive results in the coagulation assay due to the effect of the parent compound dabigatran that could have been liberated from the acylglucuronides. Dabigatran trace amounts eventually formed during the coagulation assays were quantified using a validated HPLC-tandem mass spectrometry assay (Bleich et al., 2008).

In Vitro Incubation Experiments for Dabigatran Glucuronidation. Incubation experiments for the assessment of enzyme kinetic data with liver and intestinal microsomes as well as expressed UGT1A9 and UGT2B7 were performed in 0.1 M MES buffer, pH 6.0, containing 10 mM magnesium chloride, 25 μg/ml alamethicin, saccharic acid 1,4-lactone (5 mM), and UDPGA (3 mM) in a total volume of 100 μl. The reaction was initiated by addition of UDPGA at 37°C. Control incubations were performed without UDPGA. The reaction was terminated by addition of 10 μl of hydrochloric acid (1 M) and transfer onto ice. All incubations were performed in at least duplicate. Enzyme kinetics with expressed UGT2B15 were assessed using 0.1 M Tris-HCl, pH 7.6 (pH at ambient temperature, pH 7.4 at 37°C). Quantitative HPLC Analysis of Incubation Experiments. The 1-O-acylglucuronide dabigatran that was formed during in vitro experiments was quantified by HPLC on a 150 × 4.6 mm Zorbax SB-AQ, 5 μm column, and a 12.5 × 4.6 mm guard column of the same stationary phase using a gradient of aqueous ammonium acetate (0.1 M, pH 4.5, mobile phase A) versus acetonitrile (mobile phase B) at a flow rate of 1.0 ml/min at 40°C (gradient: 90% A at 0 min, linear increase to 30% B at 8 min, step to 95% B at 8.1 min, and plateau at this composition for 1 min). Eluting peaks were detected by UV absorption at 305 nm. For incubation experiments with intestinal microsomes, a modified HPLC gradient was used because of the presence of an interfering peak: 90% A at 0 min, plateau at this composition for 4 min, linear increase to 15% B at 8 min, plateau at this composition for 2 min, step to 95% B at 10.1 min, and plateau at this composition for 2 min. The HPLC system using UV detection for the analysis of dabigatran glucuronide was validated over the range of linear detector response (concentration range of 0.005 to 50 μM). Dabigatran 1-O-acylglucuronide was not available as a synthetic reference material of defined purity; thus, dabigatran was used as a calibration standard for the HPLC. To assess the detector response and linearity, a comparison was made between calibration curves of dabigatran and radioactive dabigatran 1-O-acylglucuronide. This material had been isolated during ADME studies and was available in only very limited amounts. Because the dabigatran 1-O-acylglucuronide exhibited slightly higher UV absorption compared with dabigatran, a conversion factor of 0.837 was calculated using the slopes of the calibration curves of the dabigatran 1-O-acylglucuronide and of dabigatran. This conversion factor was used throughout the study to correct the amount of the formed dabigatran 1-O-acylglucuronide when a calibration curve obtained with dabigatran as a reference standard was used.

Enzyme Kinetics of In Vitro Glucuronidation. For the assessment of enzyme kinetics, dabigatran substrate concentrations of 0.5 to 750 μM were used (1000 μM for some experiments). After pilot incubation experiments with different concentrations of microsomal protein and different incubation times, enzyme kinetic experiments were performed at a protein concentration of 1 mg/ml and an incubation time of 45 min. All incubation experiments were performed in duplicate.

Data Analysis. Assessment of anticoagulant activity. The concentrations of dabigatran and its 1-O-acylglucuronide required to induce a doubling in clotting time (i.e., EC2) was calculated by computer-assisted nonlinear regression analysis using Biometrics Group validated methods (Boehringer Ingelheim Pharma GmbH and Co. KG).

Calculation of kinetic data of degradation of acylglucuronides. Calculations were performed by iterative nonlinear regression analysis of the measured data using the equation for first-order reaction kinetics: C = C(0) · e⁻kt using the Solver subprogram implemented in Microsoft Excel.

Enzyme kinetics. Eadie-Hofstee graphs of quantitative data of dabigatran in vitro glucuronidation were visually inspected for the presence of nonstandard hyperbolic enzyme kinetics. Some data sets were also analyzed using the Hill equation for sigmoidal enzyme kinetics and the goodness of fit was assessed statistically. Subsequently, enzyme kinetic analysis was performed by nonlinear regression using the Michaelis-Menten equation:

\[ v = \frac{V_{max} \cdot [S]}{K_m + [S]} \]

Weighted data (1/ św or 1/ św) were used for nonlinear regression analysis. The quality of data analysis was assessed by calculation of B values according to the following equation:

\[ B = \frac{1 - \text{SQ}}{\text{2(yi} - \text{ymean})} \]

where SQ is the sum of least squares, yi is the measured value, and ymean is the mean of measured values. Iterative nonlinear regression analyses were performed using the Solver subprogram implemented in Microsoft Excel. Summary statistics and other calculations were also done using Microsoft Excel.

Results

Isolation and Purification of Dabigatran Acylglucuronides. Urine samples of rhesus monkeys dosed with dabigatran were analyzed by HPLC with UV detection at 292 nm. Four peaks were identified by liquid chromatography-tandem mass spectrometry that consisted of glucuronide metabolites of dabigatran. To isolate the glucuronide metabolites of dabigatran, the urine was then subjected to solid-phase extraction on XAD 2 resin. The XAD 2 extract was subsequently used for the isolation and purification of the four glucuronide metabolite fractions by HPLC. Four chromatographically pure glucuronides were obtained, and their chemical structure was then elucidated by NMR.

NMR Structure Elucidation of Isomeric Dabigatran Acylglucuronides. The chromatographically pure acylglucuronide fractions and, for reference purposes, the parent drug dabigatran were used for 1H NMR and 1H COSY NMR measurements. β-1-O-Acylglucuronides are formed in vivo by UGTs. Because of acyl migration, the aglycon migrates from C-1 toward C-4 on the glucuronide ring, resulting in formation of the 2-, 3-, and 4-O-acylglyconides, both as α- and β-anomers. These reactions are reversible, except for the reformation of the β-1-O-acylglyconide, presumably because of the higher energy barrier in formation of the anomeric C–O bond. This was in agreement with the acquired NMR spectra that are listed in Table 1.

The chemical shifts of the β-1-O-acylglyconide showed only slight differences compared with the parent compound dabigatran. Nevertheless, five additional protons between δ 4.29 and 6.42 ppm were observed. The sequence of these five protons of the glucuronide
1H-NMR chemical shifts and coupling constants of dabigatran, dabigatran 1-O-acylglucuronide, and its rearrangement products

**Table 1**

<table>
<thead>
<tr>
<th>Atom no.</th>
<th>Dabigatran</th>
<th>1-O-Acylglucuronide</th>
<th>2-O-Acylglucuronide</th>
<th>3-O-Acylglucuronide</th>
</tr>
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<tr>
<td>H-1</td>
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<td>4.36</td>
<td>4.36</td>
</tr>
<tr>
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<td>4.77</td>
<td>4.77</td>
<td>4.77</td>
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</table>

The chemical shifts of the 2-, 3-, and 4-O-acylglucuronides are assigned with a similar approach. The results are summarized in Table 1.

**Chemical Stability and Acyl Migration of Dabigatran 1-O-Acylglucuronide.** To assess the stability of dabigatran 1-O-acylglucuronide with respect to the formation of isomeric acylglucuronides and hydrolysis to the aglycon, incubation experiments were performed in pH 7.4 aqueous buffer at 37°C. HPLC analysis was performed on samples that were taken at various time points up to 8 h. At early sampling times, the educt dabigatran 1-O-acylglucuronide was the most prominent component in the incubation mixtures (Fig. 3). Parallel to the decline in the amount of dabigatran 1-O-acylglucuronide, there was a continuous increase in the amount of the aglycon dabigatran. Isomeric acylglucuronides were formed at different rates and to different extents (Fig. 4).

Quantitative time-concentration data on the disappearance of the dabigatran 1-O-acylglucuronide were analyzed using the equation for first-order reactions by nonlinear regression analysis to assess its apparent half-life at pH 7.4. The data were well described by first-order reaction kinetics and yielded an apparent half-life of 1.0 h (Fig. 5).

**Pharmacological Activity of Dabigatran Acylglucuronides.** The anticoagulant activity in PPP of dabigatran 1-O-acylglucuronide and dabigatran was compared using the aPTT as a marker in a turbidometric assay. Baseline aPTT in control plasma was 32 s. Increasing concentrations of dabigatran resulted in prolonged aPTT values as expected. Moreover, the metabolite dabigatran 1-O-acylglucuronide showed a similar concentration-dependent prolongation of aPTT compared with the parent compound (Fig. 6). The concentrations required for a doubling in clotting time were 0.45 and 0.46 μM for dabigatran and its 1-O-acylglucuronide, respectively. In a further experiment, the effect of dabigatran 1-O-acylglucuronide on aPTT was compared with that of the three other isomeric acylglucuronides. At a concentration of 1.76 μM, all acylglucuronide isomers prolonged the aPTT in pooled platelet-poor human plasma between 4- and 5-fold over the aPTT of 34 s in control experiments. The 1-O-, 2-O-, 3-O-, and 4-O-acylglucuronides of dabigatran prolonged the aPTT to 134.4 ± 1.5, 151.5 ± 1.6, 169.4 ± 2.5, and 147.4 ± 5.3 s (mean ± S.D., n = 4), respectively. To avoid false-positive results for the acylglucuronides due to hydrolytically formed dabigatran, these samples were analyzed for the presence of dabigatran. Only small amounts of dabigatran were observed (0.1–1.1%) relative to the concentration of the acylglucuronides.

**In Vitro Dabigatran Glucuronidation.** The glucuronidation of dabigatran was investigated in vitro by using human liver or intestinal microsomes or expressed UDP-glucuronosyltransferases. The formation of dabigatran 1-O-glucuronide was assessed at a substrate concentration of 750 μM. A panel of 16 different samples of individual donors (9 male and 7 female) and two samples of pooled human liver microsomes were used to obtain information on the variability of the rate of dabigatran glucuronidation (Fig. 7). The data indicated some variability for the individual donors with three donors exhibiting a clearly higher glucuronidation rate. There was no obvious relation between the glucuronidation rate and the gender of the individual donors. One donor (no. 196) with a high rate of glucuronidation...
received medication with the enzyme inducer phenobarbital. However, another donor (no. 297), who was medicated with the enzyme inducer rifampicin, showed no elevated glucuronidation activity. There was no correlation (data not shown) between the rate of dabigatran glucuronidation and the glucuronidation activities for specific UGT test reactions (17β-estradiol 3- and 17-glucuronidation, morphine 3- and 6-glucuronidation, trifluoperazine glucuronidation, 1-naphthol glucuronidation, and propofol glucuronidation) that were provided by the supplier of the individual microsome samples. Enzyme kinetics were assessed for liver microsome samples of six (three male and three female) individual donors and intestinal microsomes of ileum and jejunum tissue of individual tissue donors. This panel of liver microsomes included the three donors with the higher glucuronidation rate. After visual inspection of the Dixon graphs and tests using standard hyperbolic and nonstandard (sigmoidal) enzyme kinetic equations, the data were analyzed by using a standard hyperbolic (Michaelis-Menten type) reaction rate equation (Table 2). Although the $K_m$ parameters of the intestinal microsomes were not dissimilar to the data for liver microsomes, the much lower $V_{\text{max}}$ values of the intestinal glucuronidation of dabigatran resulted in very low intrinsic clearance data that accounted for less than 1% of the mean of the intrinsic clearance of the individual donors of liver microsomes.

Assessment of UGTs. Dabigatran was incubated with expressed UGT enzymes in the presence of UDPGA. Two substrate concentrations of 150 and 750 μM and two different pH conditions (6.0 and 7.4) were tested to achieve sufficient product formation. Twelve different, commercially available expressed human UGT enzymes were used for incubation experiments (Fig. 8). Consistent and reproducible formation of the 1-O-acylglucuronide of dabigatran was observed only for three UGT enzymes, namely, UGT1A9, UGT2B7, and UGT2B15. UGT1A1, 1A4, 1A6, 1A7, 1A10, 2B4, and 2B17 did not show dabigatran glucuronidation. However, UGT1A3 and UGT1A8 exhibited small glucuronide formation in some experiments that was close to the analytical limit of quantification. Enzyme kinetics were then assessed at pH 6.0 for UGT1A9 and UGT2B7 and at pH 7.4 for UGT2B15 (Table 2). The glucuronidation of dabigatran by expressed human UGT enzymes followed standard hyperbolic Michaelis-Menten enzyme kinetics. However, the intrinsic clearance of the expressed UGT enzymes was much lower compared with that of human liver microsomes. This result was mainly due to the low $V_{\text{max}}$ that indicated low expression levels of the UGT. To attempt a qualification of UGTs that contribute to hepatic glucuronidation, several compounds were used that are described as inhibitors of UGT activity (Table 3). Among the nine potential inhibitors tested, no potent inhibition of hepatic dabigatran glucuronidation was observed.

It is interesting to note that ritonavir exhibited an IC$_{50}$ of 10.4 μM. This value indicated that in addition to the reported inhibition of UGT1A1, UGT1A3, and UGT1A9 (Zhang et al., 2005), ritonavir most likely also inhibits UGT2B15.

### Discussion

Glucuronidation reactions as the principal pathway for metabolic clearance are not uncommon for polar compounds that exhibit suitable structural moieties for conjugation to glucuronic acid. As such, the highly polar zwitterion dabigatran is not an exception. Indeed, the formation of the 1-O-acylglucuronide is the most prevalent biotransformation step in human and several animal species (Blech et al., 2008) with only negligible oxidative metabolism. As with other acyl-
glucuronides, the primary metabolite 1-\(O\)-acylglucuronide isomerizes by nonenzymatic acyl migration (Shipkova et al., 2003). The rate of acyl migration, assessed by the stability of the 1-\(O\)-acylglucuronide in aqueous solution, can serve as a surrogate for the chemical reactivity of acylglucuronides and provide an estimate for the extent of covalent adduct formation of the acylglucuronides with protein (Benet et al., 1993; Bischer et al., 1995). The apparent half-life of dabigatran 1-\(O\)-acylglucuronide was 1.0 h and therefore in a similar range as those of widely used nonsteroidal anti-inflammatory drugs, such as...
Enzyme kinetics (Michaelis-Menten parameters) of dabigatran glucuronidation catalyzed by human liver and human intestinal microsomes and by expressed UGT enzymes

Experiments were performed at pH 6.0 except for UGT2B15, which was incubated at pH 7.4. Donor 196 was under drug treatment with phenobarbital; donors 288 and 300 did not receive medications.

| Microsomes/UGT Enzyme | K<sub>m</sub> (μM) | V<sub>max</sub>/pmol·min⁻¹·mg⁻¹ | V<sub>max</sub>/K<sub>m</sub> (μl/min·mg) | B
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<td>215</td>
<td>0.43</td>
<td>0.998</td>
</tr>
<tr>
<td>Donor 259, female&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200</td>
<td>92.3</td>
<td>0.46</td>
<td>0.99</td>
</tr>
<tr>
<td>Donor 277, male&lt;sup&gt;a&lt;/sup&gt;</td>
<td>187</td>
<td>225</td>
<td>1.2</td>
<td>0.995</td>
</tr>
<tr>
<td>Donor 290, female&lt;sup&gt;a&lt;/sup&gt;</td>
<td>180</td>
<td>153</td>
<td>0.90</td>
<td>0.994</td>
</tr>
<tr>
<td>Donor 196, male&lt;sup&gt;a&lt;/sup&gt;</td>
<td>204</td>
<td>750</td>
<td>3.7</td>
<td>0.998</td>
</tr>
<tr>
<td>Donor 288, female&lt;sup&gt;a&lt;/sup&gt;</td>
<td>255</td>
<td>828</td>
<td>3.3</td>
<td>0.999</td>
</tr>
<tr>
<td>Donor 300, male&lt;sup&gt;a&lt;/sup&gt;</td>
<td>240</td>
<td>981</td>
<td>4.1</td>
<td>0.999</td>
</tr>
<tr>
<td>Intestine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileum, female&lt;sup&gt;a&lt;/sup&gt;</td>
<td>411</td>
<td>8.6</td>
<td>0.02</td>
<td>0.999</td>
</tr>
<tr>
<td>Ileum, male&lt;sup&gt;a&lt;/sup&gt;</td>
<td>454</td>
<td>0.7</td>
<td>0.002</td>
<td>0.995</td>
</tr>
<tr>
<td>Jejunum, female&lt;sup&gt;a&lt;/sup&gt;</td>
<td>759</td>
<td>12.9</td>
<td>0.02</td>
<td>0.998</td>
</tr>
<tr>
<td>Jejunum, male&lt;sup&gt;a&lt;/sup&gt;</td>
<td>290</td>
<td>2.2</td>
<td>0.007</td>
<td>0.996</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>371</td>
<td>1.7</td>
<td>0.004</td>
<td>0.995</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>987</td>
<td>2.0</td>
<td>0.002</td>
<td>0.994</td>
</tr>
<tr>
<td>UGT2B15</td>
<td>512</td>
<td>31.8</td>
<td>0.06</td>
<td>0.999</td>
</tr>
</tbody>
</table>

<sup>a</sup> Microsome samples from individual donors.

Dabigatran is a direct thrombin inhibitor and is hydrolyzed enzymatically to its active metabolite dabigatran 1-acylglycerol. The formation of this acylglucuronide is catalyzed by human UGT enzymes. The glucuronidation of dabigatran by expressed human UGT enzymes is shown in FIG. 8. Glucuronidation of dabigatran by expressed human UGT enzymes. Substrate concentration (150 and 750 μM) and pH of incubation buffer (6.0 and 7.4) was varied to achieve detectable glucuronide formation.

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naxopen [0.92 and 1.8 h for the (R)‑ and (S)‑enantiomers, respectively] and salicylic acid (1.3 h, metabolite of aspirin). It was longer than the half‑life of the reactive 1‑O‑acylglucuronides of some drugs that were suspected to cause immunotoxic adverse reactions, such as tolmetin (0.26 h) or zomepirac (0.45 h) (Ebner et al., 1999).

Although the reactivity of the acylglucuronides has been discussed as a determining factor for the risk of idiosyncratic adverse reactions (Bailey and Dickinson, 2003), the systemic exposure to and overall body burden of the reactive acylglucuronides should also be considered. A comparison of the systemic exposure toward acylglucuronide metabolites is provided in Table 4. Although there are only sparse pharmacokinetic data on acylglucuronide metabolites available and most were obtained after a single dose, it becomes obvious that for the drugs with the highest risk of idiosyncratic adverse reactions, such as tolmetin and zomepirac, that the reactivity and the systemic burden of the acylglucuronide metabolites is high. Zomepirac was withdrawn from the market 3 years after approval, because of a high incidence of adverse reactions including several fatalities. It ranks among the most reactive acylglucuronides and also exhibits a relatively high systemic burden indicated by its plasma AUC (Table 4) and the complete oral absorption of zomepirac (Chiou and Buehler, 2002). Unlike the other compounds listed in Table 4, for the acylglucuronide of zomepirac the plasma AUC exceeds that of the parent compound. In this respect, the exposure to the acylglucuronides of dabigatran is very low, mainly because of its limited absorption and absolute bioavailability (7%) after oral dosing (Blech et al., 2008; Stangier, 2008). Together with its lower reactivity compared with that of zomepirac or tolmetin, the low absolute systemic burden of dabigatran acylglucuronide should result in a lower relative risk of idiosyncratic adverse reactions. In addition, there was no indication of drug‑related material that was covalently bound to plasma proteins during the 14C human ADME study of dabigatran (Blech et al., 2008 and data on file). It is acknowledged that the concentrations of acylglucuronides in plasma may not reflect the concentration of acylglucuronides in organs, such as the liver, and do not directly correlate with the occurrence of covalent adducts in the plasma or the liver (Bailey and Dickinson, 1996).

There are few examples in the literature of glucuronides that exhibit pharmacological activity, such as morphine‑6‑glucuronide (Christensen and Jørgensen, 1987), mycophenolate acylglucuronide (Shipkova et al., 2001), or the acylglucuronide of retinoic acid (Becker et al., 1996). Dabigatran 1‑O‑acylglucuronide, and its rearrangement products, exhibited anticoagulant activity that was comparable to that of the parent drug. This finding can be explained by the molecular interaction of dabigatran with its target protein thrombin. The pharmacophore model of thrombin indicates a strong interaction between the highly basic benzamidine moiety of dabigatran and Asp189 of the substrate‑binding pocket of thrombin (Hauel et al., 2002). The carboxylate functional group of dabigatran has no interaction with thrombin and is directed out of its binding site. Therefore, substitution of the carboxylate moiety with an acidic glucuronic acid residue has no negative effect on the binding to thrombin. Consequently, the glucuronidation of dabigatran has no effect on the clinical efficacy of dabigatran because the acylglucuronides are pharmacologically fully active. With therapeutic dosing of dabigatran etexilate (150 mg b.i.d.), the steady‑state maximal plasma concentration of the dabigatran glucuronides in patients accounted for 12 to 14% of the total drug‑related exposure; i.e., the maximal plasma concentrations were in a range of approximately 40 to 47 nM compared with 543 nM for dabigatran (Stangier et al., 2008). Therefore, it was concluded that dabigatran itself plays the major role in its pharmacological effect after oral dosing, although the effect measured by aPTT prolongation indicated equal potency of dabigatran and its major metabolite at the therapeutically relevant plasma concentrations.

In vitro experiments showed that dabigatran undergoes glucuronidation in microsomes of human liver and intestine. However, the rate of glucuronidation was much lower in human intestinal microsomes. The K<sub>m</sub> values of the enzyme kinetic investigations were in a range of ~180 to 760 μM and indicated that dabigatran is not a high‑affinity substrate for UGT enzymes. Such relatively high K<sub>m</sub> parameters are not uncommon for UGT enzymes. Although some selected substrates may exhibit a high affinity for UGT enzymes, UGTs generally show a lower affinity for their substrates and as a consequence a lower substrate specificity compared with cytochrome P450 enzymes. There was no obvious difference between the K<sub>m</sub> of the human liver and human intestinal microsomes. Therefore, a conclusion cannot be drawn about the different UGT enzymes involved in the glucuronidation of dabigatran in the two organs on the basis of the K<sub>m</sub> data. There was an approximately 8‑ to 9‑fold variability in the intrinsic clearance among the six liver microsome samples that were assessed for the enzyme kinetic parameters.
Dabigatran glucuronidation by human liver microsomes, inhibition experiments using inhibitors of various selectivity, and comparison with published data

A dabigatran concentration of 500 μM was used for inhibition experiments.

### Table 3

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>UGT Inhibited</th>
<th>IC_{50} or K_{i}</th>
<th>Reference</th>
<th>IC_{50} for Dabigatran Glucuronidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxazepam</td>
<td>2B7</td>
<td>31–63</td>
<td>Rios and Tephly, 2002</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1A1, 2B7</td>
<td>8.8 (1A1); 18.7 (2B7)</td>
<td>T. Ebner, unpublished data&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Niflumic acid</td>
<td>1A1, 1A9, 2B7</td>
<td>51.5 (1A1); 0.038 (1A9); 83 (2B7)</td>
<td>Mano et al., 2005, 2006, 2007</td>
<td>55.7</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1A1, 1A3, 1A4</td>
<td>1.9 (1A1); 6.3 (1A3); 2.0 (1A4)</td>
<td>Zhang et al., 2005</td>
<td>10.4</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>1A1, 1A9</td>
<td>112 (1A1); 53.3, 24.2 (1A9); 6.8 (2B7)</td>
<td>Mano et al., 2005, 2006, 2007</td>
<td>155</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1A1, 1A9</td>
<td>3.3 (1A1); 31.9 (1A9); 80 (2B7)</td>
<td>Yong et al., 2005; Sampol et al., 1995</td>
<td>47.8</td>
</tr>
<tr>
<td>Hecogenin</td>
<td>1A4</td>
<td>1.5</td>
<td>Uchaipichat et al., 2006</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Codeine</td>
<td>2B7</td>
<td>168</td>
<td>Macleod et al., 1992</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Sulfipyrazone</td>
<td>1A1, 1A7, 1A9, 1A10</td>
<td>46 (1A1); 16 (1A7); 11 (1A9); 58 (1A10)</td>
<td>Uchaipichat et al., 2006</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

<sup>a</sup> IC_{50} data are displayed in italics.
<sup>b</sup> IC_{50} was assessed for inhibition of estradiol 2- and 17-glucuronidation, respectively, by human liver microsomes.

Experiments with expressed human UGT enzymes showed glucuronidation of dabigatran only for UGT1A9, UGT2B7, and UGT2B15 that exhibited consistent results over different substrate concentrations and incubation pH values. Enzyme kinetic investigations with UGT1A9, UGT2B7, and UGT2B15 revealed that dabigatran was a low-affinity substrate (K_{m} = 370–990 μM). The intrinsic clearances of the expressed enzymes for the formation of the dabigatran 1-O-acylglucuronide were much lower than those for human liver microsomes because of lower V_{max} data that were most likely indicative of the lower expression levels of the UGTs in the expression system compared with the liver. UGT2B15 exhibited an intrinsic clearance that was more than 10-fold higher than that for UGT1A9 and UGT2B7 and may therefore be considered the major catalyst for dabigatran glucuronidation. Both enzymes are known to accept a variety of compounds as substrates (Ebner and Burchell, 1993; Green et al., 1994). UGT2B15 is a major UGT isoform for the glucuronidation of estrogens and androgens. Based on the amounts of hepatic mRNA, it is one of the more prominently expressed in the liver (Izukawa et al., 2009). UGT1A9 glucuronidates a large variety of differently structured exogenous compounds. Both enzymes UGT1A9 and UGT2B15 are present not only in the liver but also in extrahepatic organs (Fisher et al., 2001).

The assessment of the contribution of individual UGTs to the overall organ glucuronidation of a substrate is difficult because of the lack (with only a very few exceptions) of specific and potent inhibitors. Nevertheless, attempts were made to elucidate the effects of potential UGT inhibitors on dabigatran glucuronidation. Consistent with the experiments with expressed UGTs, hecgonin, a specific inhibitor of UGT1A4 (Uchaipichat et al., 2006), did not inhibit the glucuronidation of dabigatran. Other compounds that were described as being at least partially specific inhibitors of UGT1A9 or UGT2B15 did not result in potent inhibition of dabigatran glucuronidation. Therefore, the relative share of the two UGTs for dabigatran glucuronidation in the liver could not be assessed.

Taken together, these results show that dabigatran acylglucuronide is a pharmacologically fully active metabolite and therefore contrib-

### Table 4

Comparison of exposure toward acylglucuronide metabolites in humans and chemical stability of 1-O-acylglucuronides

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg)</th>
<th>AUC Parent Drug (nmol × h/ml)</th>
<th>AUC Acylglucuronide (nmol × h/ml)</th>
<th>Average Therapeutic Dose (mg/day)</th>
<th>Estimated AUC Acylglucuronide at Therapeutic Dose (mg)</th>
<th>Acylglucuronide t_{1/2} at pH 7.4, 37°C (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beclobrate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>230</td>
<td>21.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100</td>
<td>21.6</td>
<td>22.7 (−), 25.7 (+)</td>
</tr>
<tr>
<td>Carprofen&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50</td>
<td>86.1</td>
<td>40.9</td>
<td>300</td>
<td>245</td>
<td>1.7 (R), 1.9 (S)</td>
</tr>
<tr>
<td>Dabigatran&lt;sup&gt;e&lt;/sup&gt;</td>
<td>150</td>
<td>2.48</td>
<td>0.36&lt;sup&gt;d&lt;/sup&gt;</td>
<td>300</td>
<td>0.7</td>
<td>1</td>
</tr>
<tr>
<td>Fenoprofen&lt;sup&gt;e&lt;/sup&gt;</td>
<td>600</td>
<td>1785</td>
<td>66.7</td>
<td>2400</td>
<td>267</td>
<td>1 (R), 2 (S)</td>
</tr>
<tr>
<td>Ibuprofen&lt;sup&gt;e&lt;/sup&gt;</td>
<td>800</td>
<td>703</td>
<td>27.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3200</td>
<td>111</td>
<td>3.3</td>
</tr>
<tr>
<td>Ketoprofen&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50</td>
<td>56.1</td>
<td>13.1</td>
<td>300</td>
<td>78.6</td>
<td>0.7 (R), 1.4 (S)</td>
</tr>
<tr>
<td>Naproxen&lt;sup&gt;f&lt;/sup&gt;</td>
<td>250</td>
<td>2553</td>
<td>30.5</td>
<td>1650</td>
<td>201</td>
<td>0.9 (R), 1.8 (S)</td>
</tr>
<tr>
<td>Tolfenac&lt;sup&gt;f&lt;/sup&gt;</td>
<td>400</td>
<td>289</td>
<td>6.30</td>
<td>1800</td>
<td>28.4</td>
<td>0.26</td>
</tr>
<tr>
<td>Zomepirac&lt;sup&gt;f&lt;/sup&gt;</td>
<td>100</td>
<td>9.4</td>
<td>10.3</td>
<td>600</td>
<td>61.8</td>
<td>0.45–0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assuming linear dose-exposure relationships.
<sup>b</sup> Data for acylglucuronide half-lives are taken from Shipkova et al. (2003) and Ebner et al. (1999).
<sup>c</sup> Data from Mayer et al. (1993).
<sup>d</sup> AUC data from repeated dose study.
<sup>e</sup> Data from Iwakawa et al. (1989).
<sup>f</sup> Dosed as dabigatran etexilate (Stangier et al., 2008).
<sup>g</sup> Data from Spahn-Langguth et al. (1992).
<sup>h</sup> Data from Castillo et al. (1995).
utes to the overall anticoagulant activity of dabigatran, albeit to a
minor extent. The glucuronide was assessed indirectly from the dif-
ference between the total dabigatran after hydrolysis of the glucuro-
nide and the free, nonconjugated dabigatran. The shapes of the plasma
concentration-time profiles of free and total dabigatran and the re-
spective half-lives were found to be identical (Stangier, 2008). Al-
though dabigatran acylglucuronide shares the chemical instability of
this compound class of metabolites, the risk of adverse reactions that
may be triggered by its chemical reactivity is considered to be small
because of its stability data and the low absolute body burden com-
pared with that of other acylglucuronides. The major contribution of
UGT2B15 and the minor contribution of at least two more UGT
enzymes together with the lack of potent inhibition of dabigatran
glucuronidation by several potential UGT inhibitors indicate a low
risk of interaction by comedICATIONS on dabigatran glucuronidation in
the clinic.

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