0090-9556/05/3310-1532–1537$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 33, No. 10
Copyright © 2005 by The American Society for Pharmacology and Experimental Therapeutics
4572/3054642
DMD 33:1532–1537, 2005
Printed in U.S.A.

# METABOLISM OF *N*-HYDROXYGUANIDINES (*N*-HYDROXYDEBRISOQUINE) IN HUMAN AND PORCINE HEPATOCYTES: REDUCTION AND FORMATION OF GLUCURONIDES

Anja Kristina Froehlich, Ulrich Girreser, and Bernd Clement

*Institute of Pharmacy, Christian-Albrechts-University of Kiel, Germany*

Received March 2, 2005; accepted July 18, 2005

**ABSTRACT:**

**The biotransformation of *N*-hydroxydebrisoquine, a model substrate for *N*-hydroxyguanidines, was studied in vitro with cultured and characterized porcine and human hepatocytes. The objective of the present work was to compare the *N*-oxidative and *N*-reductive metabolism of this compound using a monolayer culture system with previously described microsomal studies and to investigate the phase 2 metabolism, in particular, the glucuronidation of this class of compounds. At the same time, the suitability of pig hepatocytes as a model system for the human metabolism could be investigated. Two glucuronides of the parent compound *N*-hydroxydebrisoquine were analyzed. For the first time, one of these phase 2 metabolites could be identified as an *O*-glucuronide of an *N*-hydroxyguanidine by comparing it to a synthesized authentic compound. The involvement of certain human UDP-glucuronosyltransferases (UGTs) was evaluated by incubating the substrate with eight human hepatic recombinant UGT enzymes. Metabolites were determined by a newly developed LC-MS (liquid chromatography/mass spectrometry) analysis using electrospray ionization (ESI). The known microsomal reduction of the *N*-hydroxylated compound was also demonstrated with hepatocytes. The *N*-hydroxylation of the corresponding reduced compound (debrisoquine), which was previously described with microsomes, could not be detected in hepatocytes. There was no qualitative difference in the formation of the described derivatives by human and porcine hepatocytes. All phase 2 metabolites identified in hepatocyte culture were also formed by glucuronosyltransferases. In culture, the *N*-reduction of the *N*-hydroxylated substrate is the dominating reaction, indicating a predominance of *N*-reduction in vivo.**

Primary hepatocytes are widely used as an in vitro model to investigate xenobiotic metabolism, drug interactions, transport, and toxicity (MacGregor et al., 2001; McKay et al., 2002). Hepatocyte suspensions are applied for short-term studies (Bayliss et al., 1994; Steinberg et al., 1999), whereas cultured hepatocytes are a good tool for in vitro long-term examinations, to detect phase 1 and phase 2 metabolites and to show in vitro-in vivo correlations (Ulrich et al., 1995; Nussler et al., 2001; Pritchett et al., 2002; Brandon et al., 2003). Recently, the pig has become a more popular alternative in testing of new drug candidates (Zuber et al., 2002). The interest in porcine hepatocyte-based bioartificial liver and xenotransplantation is increasing (Sheil, 2002).

In this study, porcine hepatocytes were cultured and used to examine phase 1 (*N*-oxidation, *N*-reduction) and phase 2 metabolism. The same biotransformation was explored in cultured cryopreserved human hepatocytes to correlate with porcine metabolism.

Several pharmacologically active compounds contain a guanidine residue as a strongly basic functional group (Clement, 1986; Mutschler et al., 2001), e.g., debrisoquine, an antihypertensive agent. Former examinations were carried out with debrisoquine and the *N*-hydroxylated derivative (*N*-hydroxydebrisoquine) as model compounds for guanidines and *N*-hydroxyguanidines. We previously demonstrated the metabolic *N*-hydroxylation of guanidines for the substrates *N*,*N*′-diphenylguanidine (Clement and Kunze, 1993) and for debrisoquine (Clement et al., 1993) in rat and rabbit microsomes, whereas the facile retro-reduction of *N*-hydroxydebrisoquine to debrisoquine dominates in this enzyme source (Clement et al., 1993). Furthermore, C-oxidation, such as 4-hydroxylation of debrisoquine, a marker reaction of human CYP2D6, and phenolic hydroxylations are described in the literature (Mahgoub et al., 1977; Lightfoot et al., 2000).

However, neither the *N*-hydroxylation of debrisoquine nor the *N*-reduction or phase 2 metabolites of the *N*-hydroxydebrisoquine has been observed yet in a hepatocyte culture system that closely simulates in vivo conditions. Thus, we investigated conjugation reactions, especially glucuronidation, of the *N*-hydroxylated compound for the first time and compared phase 1 reactions of these two substrates in cultured pig and human hepatocytes with other enzyme sources.

UDP-glucuronosyltransferases (UGTs), localized in the endoplasmic reticulum of hepatic and extrahepatic tissue, represent a supergene family consisting of the families UGT1 and UGT2 (Mackenzie et al., 1997). UGTs catalyze the conjugation of UDP-glucuronic acid with xenobiotics and endogenous compounds in humans and other mammalian species to form polar conjugates, which can be eliminated (Miners and Mackenzie, 1991). Fifteen human UGTs have been identified so far (Tukey and Strassburg, 2000). The aim of our investigation was to identify human glucuronide conjugates of the *N*-hydroxylated guanidine by synthetic reference and examine the

Financial support by the Deutsche Forschungsgemeinschaft (DFG, Cl 56/6-3) and the Fonds der Chemischen Industrie is gratefully acknowledged.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.105.004572.

**ABBREVIATIONS:** UGT, UDP-glucuronosyltransferase; KRB, Krebs-Ringer buffer; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; ESI, electrospray ionization.

involvement of human UGTs by incubating the substrate in the presence of eight human hepatic recombinant UGT isoenzymes.

## Materials and Methods

**Chemicals and Reagents.** Human recombinant UGT isoenzymes, expressed in microsomes of insect cell infected with baculovirus (UGT Supersomes), UGT insect cell control Supersomes, UGT reaction mix (solution B) and the UGT reaction mix (solution A) were purchased from BD Gentest (San Jose, CA). Estradiol-3-glucuronidation activity was 970 pmol/(min × mg protein) for UGT1A1 and 150 pmol/(min × mg protein) for UGT1A3. UGT1A4 catalyzed trifluoperazine *N*-glucuronidation with a conversation rate of 1100 pmol/(min × mg protein). 7-Hydroxy-4-trifluoromethylcoumarin glucuronidation activity was 4000, 10,000, 1500, 1100, and 1150 pmol/(min × mg protein) for UGT1A6, UGT1A9, UGT2B4, UGT2B7, and UGT2B15, respectively. Plateable cryopreserved human hepatocytes, hepatocyte-thawing medium, hepatocyte-plating medium, and hepatocyte incubation medium were supplied by In Vitro Technologies (Baltimore, MD). Debrisoquine sulfate was obtained from Sigma (Taufkirchen, Germany). *N*-Hydroxydebrisoquine was synthesized as described by Bailey and DeGrazia (1973) using dioxane instead of *N*,*N*-dimethylformamide in the last reaction step. All spectroscopic data agreed with the literature values. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany), unless stated otherwise.

**Pig Hepatocyte Isolation, Cultivation, and Characterization.** Livers of German Landrace pigs received from a local slaughterhouse were stored in calcium-free Krebs-Ringer buffer (pH 7.4, 4°C) during transport. Perfusion started within 50 min after exsanguination. Therefore, cannulas were inserted in the veins of a liver piece. The method described by Reese and Byard (1981) was adopted but slightly modified. Two-step perfusion began with calcium-free Krebs-Ringer buffer (KRB) with 1.0 mM EDTA for 20 min, followed by KRB, containing 5 mM $CaCl_2$ and 0.6 mg/ml collagenase (type CLS II; Biochrom, Berlin, Germany) but no EDTA, for 15 min. The liver capsule was carefully dissected; the cells were suspended in KRB, filtered through gaze of nylon (150 and 80 μm), and washed twice with buffer by centrifugation at 50*g* for 4 min at 15°C. Eighty-two to 93% of the cells were viable as tested by trypan blue exclusion. After resuspension in Williams' E medium (Biochrom), supplemented with 10% fetal bovine serum (Biochrom), 2 mM L-glutamine (Biochrom), 9.6 μg/ml prednisolone, 0.014 μg/ml glucagon, and 0.16 unit/ml insulin (all from Sigma), 150 units/ml penicillin, and 150 μg/ml streptomycin (Biochrom), the hepatocytes were seeded onto collagen-coated (SERVA Electrophoresis GmbH, Heidelberg, Germany) 60-mm tissue culture plates (Sarstedt, Nümbrecht, Germany) at a density of $3 \times 10^6$ cells. Hepatocytes were maintained at 37°C in a humidified 95% air/5% $CO_2$ atmosphere. Initially, medium was renewed after 90 min and subsequently every 24 h.

During culture time, lasting 5 days, the pig hepatocytes were characterized by viability studies and metabolic assays. Cell viability was correlated with the leakage of lactate dehydrogenase into the supernatant, using the Cytotoxicity Detection Kit (LDH) (Roche Diagnostics GmbH, Mannheim, Germany) (Clement et al., 2001). 7-Ethoxycoumarin-*O*-deethylation activity was assessed by measuring 7-hydroxycoumarin (Sigma) and its conjugated D-glucuronic acid and sulfate using the technique described previously (Clement et al., 2001) based on the method of Ullrich and Weber (1972) and Fry and Bridges (1980). The *O*-deethylation of 7-ethoxyresorufin was quantified fluorimetrically as described (Clement et al., 2001) when applying the protocol of Burke and Mayer (1974) and Burke et al. (1994).

**Plating of Human Hepatocytes.** Cryopreserved human hepatocytes were rapidly thawed in a 37°C water bath, resuspended in warm hepatocyte-thawing medium, and centrifuged at 50*g* at room temperature for 5 min using the procedure recommended by In Vitro Technologies. After discarding of the supernatant, the cell pellet was resuspended in hepatocyte-plating medium, and the hepatocytes were plated on collagen-coated 60-mm Petri dishes at a density of $2 \times 10^6$ cells. The unattached cells were aspirated 4 h later, and new medium was added. Hepatocytes were placed into a 37°C, 5% $CO_2$ saturating humidity incubator. The medium was changed daily. The cryopreserved human hepatocytes were previously characterized by In Vitro Technologies.

**Incubation with Cultured Human and Pig Hepatocytes.** Incubations were performed on days 1 to 3 after culturing. Hepatocytes were washed twice with warm 0.15 M sodium chloride solution and incubated with 200 μM substrate (*N*-hydroxydebrisoquine, debrisoquine) in KRB pH 7.4 (pig hepatocytes), or in hepatocyte incubation media (human hepatocytes), respectively, for 1 or 6 h at 37°C and 5% $CO_2$ in humidified air. The incubations were stopped by aspirating the supernatant from the culture dishes, and the supernatant was frozen at −20°C and freeze-dried.

**Enzymatic Hydrolysis by β-Glucuronidase.** Incubations were performed in 1.5-ml reaction vessels in a shaking water bath at 37°C for 15 h. Then, 8 μl of β-glucuronidase from *Escherichia coli* (Roche Diagnostics GmbH, Mannheim, Germany) was added to hepatocyte incubation supernatant, which was aspirated previously, and the incubations were freeze-dried.

**Incubations with UDP-Glucuronosyltransferase.** Incubations ($n = 3$) were carried out in a shaking water bath at 37°C in 1.5-ml reaction vessels for 6 h. The standard incubation mixture (0.2 ml) consisted of substrate (*N*-hydroxydebrisoquine), protein, and UGT reaction mix solutions A and B, resulting in final concentrations of 2 mM uridine 5′-diphosphoglucuronic acid, 50 mM Tris-HCl buffer pH 7.5, 10 mM $MgCl_2$, 50 μM substrate, and 25 μg/ml alamethicin. The following final protein concentrations were applied: for UGT1A1, 1A3, and UGT insect cell control Supersomes, 1.0 mg/ml; for UGT1A6, 1A9, 2B4, 2B7, and 2B15, 0.25 mg/ml. Substrates were dissolved in dimethyl sulfoxide with a maximal final concentration of 0.25%. After incubation, reactions were stopped by freezing at −20°C, and freeze drying followed afterward.

**HPLC Method for the Determination of *N*-Hydroxydebrisoquine *O*-Glucuronide and Debrisoquine.** Freeze-dried samples were resolved in 90 μl (hepatocytes incubation sample) or in 40 μl (UGT incubation sample) of mobile phase, respectively. After mixing and centrifugation, the clear supernatant was analyzed using a HP 1090 Series II high-performance liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a variable wavelength UV detector (HP VWD 1050) set at 208 nm. Separations were performed at room temperature by gradient elution on a reverse phase column (250 × 4-mm i.d., particle size, 5 μm; Lichrospher RP-select B; Merck), with a 4 × 4-mm guard column of the same material. Mobile phase A consisted of 25 mM ammonium formate buffer (pH 4.0) and acetonitrile (95:5, v/v), and mobile phase B of pure acetonitrile. Elution started with 100% mobile phase A, linearly changing to 80% mobile phase A and 20% mobile phase B from 0 to 30 min, returning to 100% mobile phase A from 35 to 40 min, and equilibrating followed from 40 to 50 min. The eluent was passed through the column at a rate of 0.5 ml/min. The solvents used in the analysis were filtered through a Sartolon membrane filter (0.45 μm; Sartorius AG, Göttingen, Germany) and degassed by sonification or bubbling with helium. Aliquots of 20 μl were injected. The retention times were 29.4 ± 0.7 min (*N*-hydroxydebrisoquine) and 31.6 ± 0.5 min (debrisoquine).

**HPLC Method for the Determination of Oxidation Products of Debrisoquine.** HPLC analysis of debrisoquine oxidation was performed with the same stationary phase as described above with following changes. Mobile phase A consisted of 25 mM ammonium formate buffer (pH 4.0) and acetonitrile (93:7, v/v), and mobile phase B of pure acetonitrile. The following gradient was used: 100% mobile phase A from 0 to 65 min, linearly changing to 80% mobile phase A and 20% acetonitrile from 65 to 70 min, linearly changing was continued to 100% mobile phase A from 75 to 85 min, and equilibrating prior next injection. The retention time of *N*-hydroxydebrisoquine was 53.0 ± 0.5 min.

**Mass Spectra of Metabolites by LC/MS Coupling.** For mass spectrometric analysis, the same conditions were used as mentioned above for HPLC analysis, with a split of 1:6 to the mass spectrometer. Electrospray ionization (ESI) mass spectra were recorded in the positive ion-mode on an Esquire LC mass spectrometer (Bruker, Bremen, Germany). Synthetic standards were analyzed in comparison to the metabolites of the incubations. For *N*-hydroxydebrisoquine, ions with *m/z* 192 $[M+H]^+$, 175 $[M + H - NH_3]^+$, and 132 $[M+H - HCNH_2NOH]^+$ were found. For debrisoquine, ions with *m/z* 176 $[M+H]^+$, 159 $[M+H - NH_3]^+$, and 134 $[M + 2H - CNH_2NH]^+$ were characteristic. The incubations were assessed in the full scan mode in the range of *m/z* 50 to 800 to detect any other possible metabolites.

**Synthesis of *N*-Hydroxydebrisoquine *O*-Glucuronide.** *N*-Hydroxydebrisoquine (0.96 g; 5.0 mmol) (**2**) and 1.72 g (10.0 mmol) of cadmium carbonate were suspended in 75 ml of dried toluene. Methyl-(2,3,4-tri-*O*-acetyl-1-bromo-1-desoxy-α-D-glucopyranosyl)-uronate (3.97 g; 10.0 mmol) (**1**) (Bollenback et al., 1955), dissolved in 50 ml of toluene, was added slowly to the suspension under nitrogen while distilling off the same amount of

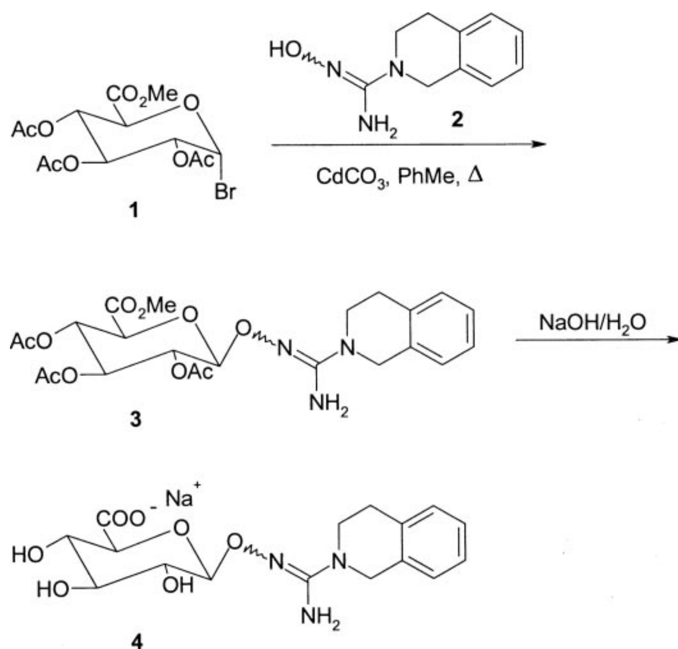


FIG. 1. Synthesis of *N*-hydroxydebrisoquine *O*-glucuronide.

solvent (Fig. 1). Subsequently, the cooled reaction mixture (room temperature) was filtered, and the solvent was evaporated in vacuum. Methanol was added to the residue, and the undissolvable particles were removed by centrifugation. The supernatant was purified by column chromatography on reversed-phase silica gel with a mixture of methanol/demineralized water (50:50, v/v), followed by flash column chromatography using a gradient of water and methanol. The yield of (methyl{2,3,4-tri-*O*-acetyl-1-*O*-[3,4-dihydro-2(1*H*)-isochinoline-carboxamidoxime]-β-D-glucuopyranosyl}-uronate) (**3**) was 4 mg (8 μmol; 0.2%) with the following spectroscopic data: $^1$H NMR (300 MHz, $CDCl_3$) δ 1.94/2.03/2.05 (3 × s, 3 × 3 H, $CH_3$); 2.88 (t, 2 H, $J$ = 5.6 Hz, $H_{aliphat.}$); 3.43 (t, 2 H, $J$ = 5.8 Hz, $H_{aliphat.}$); 3.74 (s, 3 H, $OCH_3$); 4.13 (d, 1 H, $J$ = 9.33 Hz, H-5); 4.33 (s, 2 H, $H_{aliphat.}$); 5.01 (d, 1 H, $J$ = 8.2 Hz, H-1); 5.24 (m, 3 H, H-2/H-3/H-4); 7.13 (m, 4 H, $H_{arom.}$-5′/6′/7′/8′); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 21.2/21.3/21.5 (Me); 29.5 ($C_{aliphat.}$); 44.4 ($C_{aliphat.}$); 48.4 ($C_{aliphat.}$); 53.5 (OMe); 70.4 (C-5); 70.8 (C-4); 72.9 (C-3); 73.4 (C-2); 102.8 (C-1); 126.8/127.1/134.2/135.0 ($C_{arom.}$-5′/6′/7′/8′); 158.8 (C = N); 168.0/170.1/170.4/170.7 (C = O); MS (ESI) *m/z* 508 $[M+H]^+$.

The obtained compound solved in methanol was treated with an aqueous sodium hydroxide solution at room temperature for 30 min. After complete splitting off of the protection groups, the product 1-*O*-[3,4-dihydro-2(1*H*)-isochinolinecarboxamidoxime]-β-D-glucuopyranosyl-uronate (*N*-hydroxydebrisoquine *O*-glucuronide) (**4**) was analyzed immediately by LC/MS analyses and showed ions with *m/z* 368 $[M+H]^+$, 390 $[M+Na]^+$, 406 $[M+K]^+$, 412 $[M - H + 2Na]^+$, 428 $[M - H+Na+K]^+$, and 174 [*N*-hydroxydebrisoquine-$H_2O]^+$ at a retention time of 22.5 ± 0.3 min (Fig. 2).

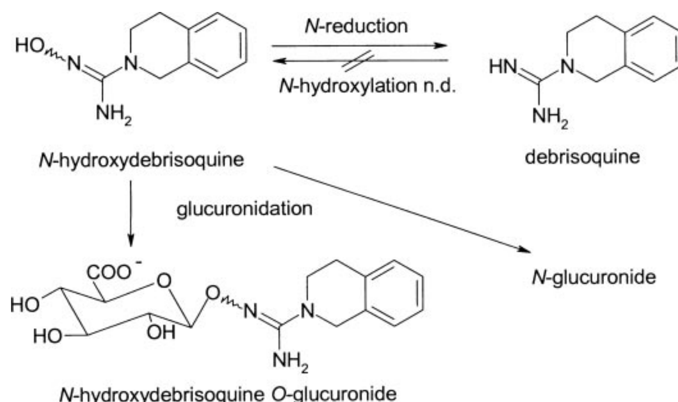


FIG. 3. *N*-Reduction of *N*-hydroxydebrisoquine by cultured human and porcine hepatocytes. Reverse *N*-hydroxylation of debrisoquine to *N*-hydroxydebrisoquine by cultured human and porcine hepatocytes is not detectable (n.d.). Shown is the formation of two different glucuronides of *N*-hydroxydebrisoquine by human and porcine hepatocytes and UDP-glucuronosyltransferases.

## Results

**Isolation, Cultivation, and Characterization of Pig Hepatocytes.** Pig hepatocytes were isolated and cultivated for 4 to 5 days. The results of hepatocyte characterization correspond to literature data (Clement et al., 2001). Thus, the described incubations were carried out during the first 3 days.

**Metabolism of *N*-Hydroxydebrisoquine in Hepatocytes.** Incubations of *N*-hydroxydebrisoquine in porcine and human hepatocytes lead to the *N*-reduced debrisoquine to a great extent (Fig. 3). Retention time and fragmentation of the metabolite agrees with those of the reference compound (Fig. 4). Incubations without hepatocytes do not show this metabolite (data not shown).

Furthermore, the incubations with hepatocytes from both species result in formation of two glucuronides of *N*-hydroxydebrisoquine.

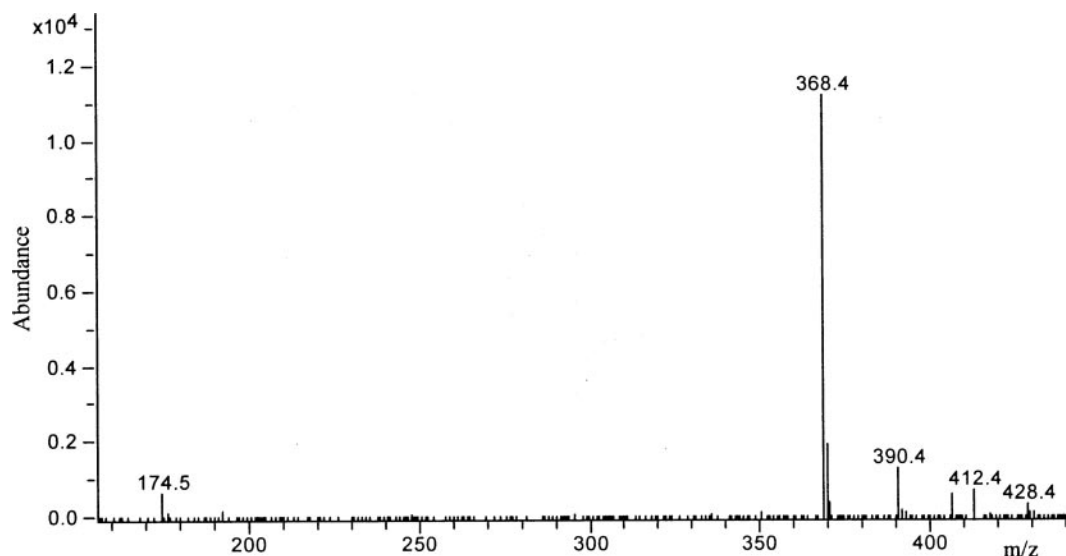


FIG. 2. Representative mass spectrum of the synthetic *N*-hydroxydebrisoquine *O*-glucuronide with a retention time of 22.5 ± 0.3 min.

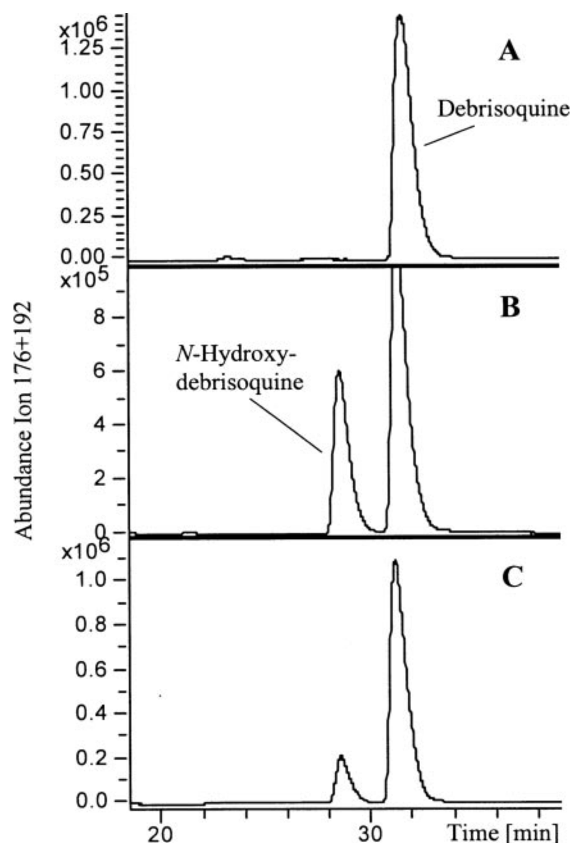


FIG. 4. Representative ion chromatograms for the signal with *m/z* 192 + 176 as characteristic ions of *N*-hydroxydebrisoquine (*m/z* 192) with a retention time of 29.4 ± 0.7 min and debrisoquine (*m/z* 176) with a retention time of 31.6 ± 0.5 min, showing *N*-reduction of *N*-hydroxydebrisoquine. A, debrisoquine as reference compound; B, incubation with cultured human hepatocytes; and C, incubation with porcine hepatocytes.

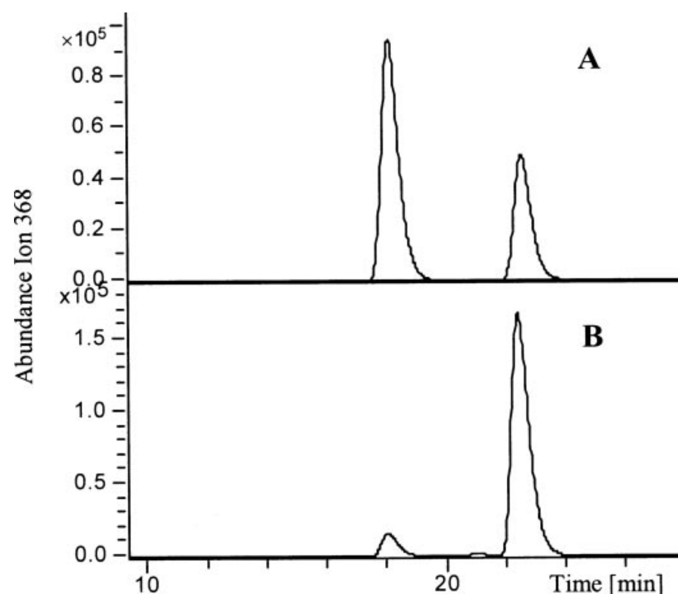


FIG. 5. Representative ion chromatograms for the signal at *m/z* 368 as characteristic ion of *N*-hydroxydebrisoquine glucuronides with retention times of 18.0 ± 0.2 min and 22.5 ± 0.3 min. A, incubation with cultured human hepatocytes; and B, incubation with porcine hepatocytes.

The retention times of these metabolites are 18.0 ± 0.2 and 22.5 ± 0.3 min. The dominant ion is at *m/z* 368 $[M+H]^+$ of the parent compound. The *m/z* 390 $[M+Na]^+$, 406 $[M+K]^+$, 412 $[M-H+2Na]^+$, and 428 $[M-H+Na+K]^+$ represent cluster of the glucuronides associated with sodium and potassium ions. The ion at *m/z* 192 indicates the loss of the glucuronic group of the parent compound. Representative ion chromatograms are shown in Fig. 5. Pig and human hepatocytes formed the same glucuronides, however, in different ratios. Incubations without hepatocytes do not show these metabolites (data not shown). After filling the hepatocyte incubation supernatant in reaction vessels, β-glucuronidase caused a hydrolysis of the glucuronide eluted after 22.5 min, which led to detectable formation of *N*-hydroxydebrisoquine. β-Glucuronidase did not decompose the supposed hydroxydebrisoquine glucuronide with the retention time of 18.0 min. Other phase 2 metabolites, such as sulfates or acetyl conjugates, could not be detected in cultures of human and porcine hepatocytes (data not shown).

**Metabolism of *N*-Hydroxydebrisoquine with UDP-Glucuronosyltransferase.** *N*-Hydroxydebrisoquine glucuronidation activity of UGT isoenzymes was measured using commercially available hepatic recombinant human UGT isoforms, including UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, and 2B15. Glucuronidation of the *N*-hydroxyguanidine was not detected after incubation with UGT1A6 and 2B4. UGT2B15 formed only the *N*-hydroxydebrisoquine glucuronide eluted after 22.5 min. Incubations with all other isoforms revealed both glucuronides by LC/MS. In consequence of the absence of a sufficient amount of the reference compound, conversion rates were referred to integrated area of the respective glucuronide related to incubation time and protein content. The resulting rankings (Student's *t* test, $p < 0.05$) in order of *N*-hydroxydebrisoquine glucuronidation activity are UGT1A9 > UGT1A4 ≈ UGT1A1 > UGT2B7 ≈ UGT1A3 for the metabolite eluted after 18.0 min and UGT1A9 > UGT1A1 > UGT1A3 ≈ UGT2B7 ≈ UGT1A4 ≈ UGT2B15 for the glucuronide with the retention time of 22.0 min. A representative ion chromatogram of a *N*-hydroxydebrisoquine incubation with UDP-glucuronosyltransferases for the signal at *m/z* 368 is shown in Fig. 6. Again, the glucuronide with the retention time of 22.5 min is the only derivative that was hydrolyzed by β-glucuronidase (Fig. 6). Incubations with UGT insect cell control Supersomes did not form the described glucuronides.

**Metabolism of Debrisoquine in Hepatocytes.** The *N*-hydroxylation of debrisoquine to *N*-hydroxydebrisoquine in porcine and human hepatocytes was not detectable by LC/MS analysis (Fig. 3). No metabolite was formed with identical retention time and mass spectrum as the reference substance recorded under the same conditions. Furthermore, hepatocyte incubation samples treated with β-glucuronidase did not differ from those without addition of β-glucuronidase. Therefore, *N*-hydroxydebrisoquine was detected neither directly nor after hydrolysis of potentially formed *N*-hydroxydebrisoquine glucuronide. Direct phase 2 metabolites of debrisoquine such as *N*-glucuronides, *N*-sulfates, or *N*-acetyl conjugates were not found in cultures of human and porcine hepatocytes by LC/MS analysis (data not shown).

**Synthesis of *N*-Hydroxydebrisoquine *O*-Glucuronide.** The *O*-glucuronide of *N*-hydroxydebrisoquine was synthesized and characterized in its protected form for the first time. The free glucuronide proved to be quite unstable but could be analyzed by LC/MS analyses for comparison purposes. During the synthesis of the protected glucuronide several products were formed. Thus, yields were very low.

## Discussion

The metabolic cycle of the model compound *N*-hydroxydebrisoquine and debrisoquine was mainly investigated with microsomes, 9000*g* supernatants, and isolated enzymes (Clement et al., 1993),

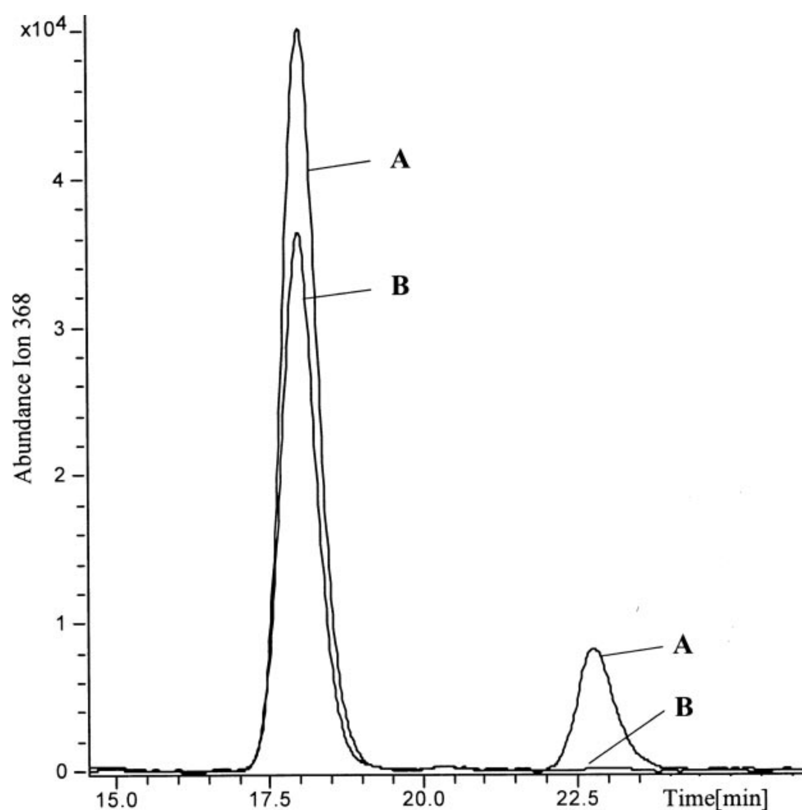


FIG. 6. Representative ion chromatograms for the signal at *m/z* 368 as characteristic ion of *N*-hydroxydebrisoquine glucuronides with retention times of 18.0 ± 0.2 min and 22.5 ± 0.3 min. A, UGT1A9 incubation; and B, UGT1A9 incubation after treatment with β-glucuronidase.

whereas this is the first study of phase 1 metabolism of *N*-hydroxydebrisoquine and of the *N*-oxidation of debrisoquine using a more complex in vitro system that simulates the in vivo situation. Cultured pig and human hepatocytes easily reduce this *N*-hydroxyguanidine to the corresponding guanidine, whereas we could not find *N*-oxidation of debrisoquine, either directly in debrisoquine incubations or after treatment with β-glucuronidase. The reduction is, therefore, the dominating reaction. The nonobservable *N*-oxidation in hepatocyte cultures might be explained by much higher conversion rates of retroreduction than those of oxidation in living, intact hepatocytes compared with microsomes. This suggests the conclusion that the *N*-reduction may dominate in vivo as well.

For the first time, we investigate phase 2 metabolism of *N*-hydroxydebrisoquine as a model compound for *N*-hydroxyguanidines. We demonstrated the formation of two different glucuronides in human and porcine hepatocyte culture by LC/MS analysis. Formation of identical glucuronides by diverse UGT isoenzymes confirmed these conjugations. The coupling product of *N*-hydroxydebrisoquine was independently synthesized using the method describing a synthesis of analog *O*-glucuronides of amidoximes (Clement et al., 2001) and was characterized by $^{1}$H NMR and $^{13}$C NMR. The absence of the signal of the *N*-hydroxy group in the $^{1}$H NMR spectra led to identification as a protected intermediate of *N*-hydroxydebrisoquine *O*-glucuronide. The formation of an *O*-glucuronide and not a *N*-glucuronide was also derived from $^{13}$C NMR data. The chemical shift of C-1 of the glucuronic acid (δ 102.8) was in concordance with the values in the range of 100 to 105 ppm previously described for *O*-glucuronides (Clement et al., 2001; Schämann and Schäfer, 2003). In the case of an *N*-glucuronide or *N*-glycosylate, a chemical shift of 80 to 90 ppm would be expected (Hoos et al., 1996; Murphy et al., 2003; Schämann and Schäfer, 2003). After cleavage of the methyl and ethyl ester, the synthetic compound showed the same retention time and mass spectrum as the phase 2 metabolites of *N*-hydroxydebrisoquine eluted after 22.5 ± 0.3 min. Furthermore, only the last eluted glucuronide formed by hepatocytes and UGTs was hydrolyzed by β-glucuronidase. However, the glucuronide eluted at 18.0 ± 0.2 min is stable under the described conditions. Zenser et al. (1999) published that β-glucuronidase extracted from *E. coli* preferentially hydrolyzes *O*-glucuronides in comparison to *N*-glucuronides and Yuan et al. (2002) described as well that the *N*-glucuronide of valdecoxib could not be hydrolyzed enzymatically, whereas aglycons of *O*-glucuronides were detectable. Both studies underline that the glucuronide capable of hydrolysation by β-glucuronidase is conjugated at the oxygen atom (retention time 22.5 ± 0.3 min) and support our conclusion that the metabolite that was not enzymatically decomposable may be characterized as an *N*-glucuronide of *N*-hydroxydebrisoquine. The formation of an *N*-glucuronide is emphasized by the fact that the involved UGT1A9, UGT1A4, UGT1A1, and UGT1A3 are known to glucuronidate functional groups containing nitrogen in general (Green and Tephly, 1998; Radominska-Pandya et al., 1999; Ritter, 2000; Malfatti and Felton, 2001). However, the results of this investigation are not sufficient to predict the molecular position of *N*-glucuronidation of the investigated *N*-hydroxyguanidine. We were not able to synthesize an *N*-glucuronide of *N*-hydroxydebrisoquine despite several attempts.

In the current study, the major involvement of the recombinant hepatic UGT1A9 in *N*-hydroxydebrisoquine *O*-glucuronidation in humans was demonstrated. Therefore, according to earlier studies with *N*-hydroxylated amines (Malfatti and Felton, 2001; Yueh et al., 2001) and *N*-hydroxyamidines (Fröhlich et al., 2005), the *O*-glucuronidation of *N*-hydroxyguanidines seems to be favorable catalyzed by UGT1A9 and UGT1A1.

Previous studies have demonstrated the usefulness of hepatocyte

cultures as an in vitro model for investigating metabolism of xenobiotic compounds (Nussler et al., 2001; Brandon et al., 2003); thus, we carried out incubations with characterized hepatocyte cultures during the first 3 days. Porcine and human hepatocytes formed the same major metabolites of *N*-hydroxydebrisoquine and did not *N*-hydroxylate the substrate debrisoquine. Thus, in vitro phase 1 and 2 metabolism of these two species seems to be qualitatively comparable with respect to the investigated metabolic pathways. This emphasizes that cultured porcine hepatocytes might be a suitable alternative to human hepatocytes for studying metabolism of xenobiotics in vitro.

Other metabolisms, such as 4-hydroxydebrisoquine and phenolic derivatives, were not analyzed in detail as the objective of this study was to investigate the metabolic cycle of *N*-hydroxylation and reduction of guanidines. In previous studies, we could also show that in microsomal systems *N*-hydroxydebrisoquine is further transformed to the urea derivative (Clement et al., 1993) and the cyanamide analog (Clement et al., 1999), resembling the metabolism of *N*-hydroxyarginine to nitric oxide and citrulline. In this study optimized for the detection of the bioreversible *N*-hydroxylation of debrisoquine and reduction of *N*-hydroxydebrisoquine, there was no evidence for the formation of nitric oxide and the urea and cyanamide derivative of debrisoquine. However, this has to be verified by further studies using this hepatocyte culture model.

In summary, the *N*-reduction of the *N*-hydroxylated substrate is the dominating reaction, whereas *N*-oxidation of debrisoquine was not observed. Parallel to this, the formation of regioisomeric glucuronides is found as the major phase 2 reaction of *N*-hydroxydebrisoquine. By comparing to a newly synthesized reference compound, an *O*-linked conjugated *N*-hydroxydebrisoquine glucuronide was identified. UGT1A9 seems to be the mainly involved enzyme in the *O*-glucuronidation of *N*-hydroxyguanidine.

## References


Bailey DM and DeGrazia CG (1973) Hydroxyguanidines. A new class of antihypertensive agents. *J Med Chem* **16:**151–156.

Bayliss MK, Bell JA, Wilson K, and Park GR (1994) 7-Ethoxycoumarin *O*-deethylase kinetics in isolated rat, dog and human hepatocyte suspension. *Xenobiotica* **24:**231–241.

Bollenback GN, Long JW, Benjamin DG, and Lindquist JA (1955) The synthesis of aryl-D-glucopyranosiduronic acids. *J Am Chem Soc* **77:**3310–3315.

Brandon EFA, Raap CD, Meijerman I, Beijnen JH, and Schellens JHM (2003) An update on in vitro test methods in human hepatic drug biotransformation research: pros and contras. *Toxicol Appl Pharmacol* **15:**233–246.

Burke MD and Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos* **2:**583–588.

Burke MD, Thompson S, Weaver RJ, Wolf CR, and Mayer RT (1994) Cytochrome P450 specificities of alkoxyresorufin *O*-dealkylation in human and rat liver. *Biochem Pharmacol* **48:**923–936.

Clement B (1986) Biotransformation stark basischer funktioneller Gruppen durch Stickstoffoxidation, in *Hochschulsammlung Naturwissenschaft Chemie und Pharmazie Band* 3, Hochschulverlag, Freiburg.

Clement B, Boucher J-L, Mansuy D, and Harsdorf A (1999) Microsomal formation of nitric oxide and cyanamides from non-physiological *N*-hydroxyguanidines: *N*-hydroxydebrisoquine as a model substrate. *Biochem Pharmacol* **58:**439–445.

Clement B, Christiansen K, and Girreser U (2001) Phase 2 metabolites of *N*-hydroxylated amidines (amidoximes): synthesis, in vitro formation by pig hepatocytes and mutagenicity testing. *Chem Res Toxicol* **14:**319–326.

Clement B and Kunze T (1993) In vitro oxygenation of *N,N'*-diphenylguanidines. *Xenobiotica* **23:**155–167.

Clement B, Schultze-Mosgau M-H, and Wohlers H (1993) Cytochrome P450 dependent *N*-hydroxylation of a guanidine (debrisoquine), microsomal catalysed reduction and further oxidation of the *N*-hydroxy-guanidine metabolite to the urea derivative. Similarity with the oxidation of arginine to citrulline and nitric oxide. *Biochem Pharmacol* **46:**2249–2267.

Fröhlich AK, Girreser U, and Clement B (2005) Metabolism of benzamidoxime (*N*-hydroxyamidine) in human hepatocytes and role of UDP-glucuronosyltransferases. *Xenobiotica* **35:**17–25.

Fry JR and Bridges JW (1980) The metabolism of 7-ethoxycoumarin in primary maintenance cultures of adult rat hepatocytes. *Naunyn-Schmiedeberg's Arch Pharmacol* **311:**85–90.

Green MD and Tephly TR (1998) Glucuronidation of amine substrates by purified and expressed UDP-glucuronosyltransferase proteins. *Drug Metab Dispos* **26:**860–867.

Hoos R, Huixin J, Vasella A, and Weiss P (1996) Synthesis and enzymatic evaluation of substrates and inhibitors of $\beta$-glucuronidases. *Helv Chim Acta* **79:**1757–1784.

Lightfoot T, Ellis SW, Mahling J, Ackland MJ, Blaney FE, Bijloo GJ, De Groot MJ, Vermeulen NP, Blackburn GM, Lennard MS, and Tucker GT (2000) Regioselective hydroxylation of debrisoquine by cytochrome P4502D6: implications for active site modelling. *Xenobiotica* **30:**219–233.

MacGregor JT, Collins JM, Sugiyama Y, Tyson CA, Denan J, Smith L, Andersen M, Curren RD, Hoeutson JB, Kadlubar F, et al. (2001) In vitro human tissue models in risk assessment: report of a consensus-building workshop. *Toxicol Sci* **59:**17–36.

Mackenzie PIO, Burchell IS, Bock B, Bairoch KW, Bélanger A, Fournel-Gigleux A, Green S, Hum M, Iyanagi DW, Lancet T, et al. (1997) The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* **7:**255–269.

Mahgoub A, Idle JR, Dring LG, Lancaster R, and Smith RL (1977) Polymorphic hydroxylation of debrisoquine in man. *Lancet* **2:**584–586.

Malfatti MA and Felton JS (2001) *N*-Glucuronidation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pydidine (PhIP) and *N*-hydroxy-PhIP by specific human UDP-glucuronosyltransferases. *Carcinogenesis* **22:**1087–1093.

McKay GC, Henderson C, Goldie E, Connel G, Westmoreland C, and Grant MH (2002) Cryopreservation of rat hepatocytes monolayers: cell viability and cytochrome P450 content in post-thaw cultures. *Toxicol In Vitro* **16:**71–79.

Miners JO and Mackenzie PI (1991) Drug glucuronidation in humans. *Pharmacol Ther* **51:**347–369.

Murphy PV, Bradley H, Tosin M, Pitt N, Fitzpatrick GM, and Glass WK (2003) Development of carbohydrate-based scaffolds for restricted presentation of recognition groups. Extention to divalent ligands and implications for the structure of dimerized receptors. *J Org Chem* **68:**5692–5704.

Mutschler E, Geisslinger G, Kroemer HK, and Schäfer-Korting M (2001) *Arzneimittelwirkungen: Lehrbuch der Pharmakologie und Toxikologie.* Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart.

Nussler AK, Wang A, Neuhaus P, Fischer J, Yuan J, Liu L, Zeilinger K, Gerlach J, Arnold PJ, and Albrecht W (2001) The suitability of hepatocyte culture models to study various aspects of drug metabolism. *Altex* **18:**91–101.

Pritchett JJ, Kuester RK, and Sipes IG (2002) Metabolism of bisphenol a in primary cultured hepatocytes from mice, rats and humans. *Drug Metab Dispos* **30:**1180–1185.

Radominska-Pandya A, Czernik PJ, Little JM, Battaglia E, and Mackenzie PI (1999) Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metab Rev* **31:**817–899.

Reese JA and Byard JL (1981) Isolation and culture of adult hepatocytes from liver biopsies. *In Vitro* **17:**935–940.

Ritter JK (2000) Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. *Chem-Biol Interact* **129:**171–193.

Schämann M and Schäfer HJ (2003) TEMPO-mediated anodic oxidation of methyl glycosides and 1-methyl and 1-azido disaccharides. *Eur J Org Chem* **2:**351–358.

Sheil AG (2002) Xenogeneic bioartificial liver support: where are we now? *Transplant Proc* **34:**2493–2495.

Steinberg P, Fischer T, Kiulies S, Biefang K, Platt K-L, Oesch F, Böttger T, Bulitta C, Kempf P, and Hengstler J (1999) Drug metabolizing capacity of cryopreserved human, rat and mouse liver parenchymal cells in suspension. *Drug Metab Dispos* **27:**1415–1422.

Tukey RH and Strassburg CP (2000) Human UDP-glucuronosyltransferases: metabolism, expression and disease. *Ann Rev Pharmacol Toxicol* **40:**581–616.

Ullrich V and Weber P (1972) The *O*-dealkylation of 7-ethoxycoumarin by liver microsomes. *Hoppe-Seyler's Z Physiol Chem* **353:**1171–1177.

Ulrich RG, Bacon JA, Cramer CT, Peng GW, Petrella DK, Stryd RP, and Sun EL (1995) Cultured hepatocytes as investigational models for hepatic toxicology: practical applications in drug discovery and development. *Toxicol Lett* **82/83:**107–115.

Yuan JJ, Yang DC, Zhang JY, Bible R Jr, Karim A, and Findlay JW (2002) Disposition of a specific cyclooxygenase-2 inhibitor, valdecoxib, in human. *Drug Metab Dispos* **30:**1013–1021.

Yueh M-F, Nguyen N, Famourzadeh M, Strassburg CP, Oda Y, Guengerich FP, and Tukey RH (2001) The contribution of UDP-glucuronosyltransferase 1A9 on CYP1A2-mediated genotoxicity by aromatic and heterocyclic amines. *Carcinogenesis* **22:**943–950.

Zenser TV, Lakshmi VM, and Davis BB (1999) Human and *Escherichia coli* beta-glucuronidase hydrolysis of glucuronide conjugates of benzidine and 4-aminobiphenyl and their hydroxy metabolites. *Drug Metab Dispos* **27:**1064–1067.

Zuber R, Anzenbacherova E, and Anzenbacher P (2002) Cytochromes P450 and experimental models of drug metabolism. *J Cell Mol Med* **6:**189–198.


**Address correspondence to:** Bernd Clement, Pharmazeutisches Institut, Christian-Albrechts-Universität Kiel, Gutenbergstr. 76, D-24118 Kiel, Germany. E-mail: bclement@pharmazie.uni-kiel.de