CHARACTERIZATION OF THE UDP-GLUCURONOSYLTRANSFERASES INVOLVED IN THE GLUCURONIDATION OF AN ANTITHROMBOTIC THIOXYLOSIDE IN RAT AND HUMANS

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ABSTRACT:

To investigate the glucuronidation on the hydroxyl group of carbohydrate-containing drugs, in the vitro formation of glucuronides on the thioxyloside ring of the antithrombotic drug, LF 4.0212, was followed in rat and human liver microsomes and with recombinant UDP-glucuronosyltransferases (UGT). The reaction revealed a marked regioselectivity in rat and humans. Human liver microsomes glucuronidated the compound mainly on the 2-hydroxyl position of the thioxyloside ring, whereas rat was able to form glucuronide on either the 2-, 3-, or 4-hydroxyl group of the molecule, although to a lower extent. LF 4.0212 was a much better substrate of human UGT than the rat enzyme (V_max/K_m 30.0 and 0.06 μl/min/mg, respectively). Phenobarbital, 3-methylcholanthrene, and clofibrate enhanced the glucuronidation of LF 4.0212 on positions 2, 3, and 4 of the thioxyloside ring, thus indicating that several UGT isoforms were involved in this process. The biosynthesis of the 2-O-glucuronide isomer was catalyzed by the human UGT1A9 and 2B4, but not by UGT1A6 and 2B11. By contrast, the rat liver recombinant UGT1A6 and 2B1 failed to form the 2-O-glucuronide isomers. From all the recombinant UGTs tested, none catalyzed the formation of the 3-O-glucuronide isomer. Interestingly, glucuronidation on the 4-position was found in all the metabolic competent V79 cell lines considered, including the nontransfected V79 cells, suggesting the presence of an endogenous UGT in fibroblasts able to actively glucuronidate the drug. This activity, which was nonsensitive to the inhibitory effect of 7,7,7-triphenyl-heptanoic acid, a potent UGT inhibitor, could reflect the existence of a different enzyme.

UDP-glucuronosyltransferases (UGT, EC 2.4.1.17) are a multigeneic family of proteins actively involved in the final metabolism of a large variety of structurally unrelated substances. Drugs, pollutants, and toxic chemicals present in our environment are substrates of the enzymes. At least 70 cDNAs encoding animal and human UGTs have been isolated to date (Mackenzie et al., 1997). These enzymes catalyze the transfer of glucuronic acid from the high-energy donor, UDP-glucuronic acid, to the hydroxyl, carboxyl, amine, or thiol group of compounds, leading to the formation of water-soluble glucuronides that are excreted into bile or urine. UGTs are also actively involved in the metabolism of key endogenous substances (steroids, thyroid hormones, retinoids; Nebert, 1991). They regulate the concentration of these ligands, which participate to cell differentiation and growth.

From all substrates investigated so far, the molecules containing a carbohydrate moiety, such as the nucleoside analog antiviral drugs, constitute a peculiar class of compounds. The binding of glucuronic acid on the free hydroxyl groups of the hydrocarbon chain by glycosidic bonds between two carbohydrate entities leads to the formation of heteroside-like compounds. This is the case of the anti-HIV drug 3’-azido-3’-deoxythymidine (AZT), which is glucuronidated on the 5’-O of the ribose in humans (Haumont et al., 1990; Rajaonarison et al., 1991). The glucuronidation reaction leads to a pharmacologically inactive metabolite, which impairs its therapeutic efficiency. The same situation has also been reported for the nucleoside analog carbovir (Patanella et al., 1990). On the other hand, UGTs have been reported to catalyze the addition of a glucuronic acid group on endogenous compounds such as glycosides, glycolipids, or glycosaminoglycans (Chou et al., 1991; Lacarelle et al., 1993; Castle, 1993) and heparins (Lidholt and Lindahl, 1992). The function of these UGTs is beginning to be explored.

Until now, among the UGTs characterized, some of them in our laboratory, such as UGT2B4 or UGT2B1 (Fournel-Gigleux et al., 1991; Pritchard et al., 1994) no one has been reported to glucuronidate to an appreciable extent class of glycosidic drugs. This study is therefore aimed to better understand glucuronidation on the hydroxyl group of carbohydrate-containing drugs and to determine the UGT isoforms involved in that process. The antithrombotic drug, LF 4.0212 (4-cyanophenyl 1,5 dithio-β-d-xylopyranoside), has been used as a model substrate (Fig. 1). This compound has been found to be a good acceptor of galactose transfer and, therefore, initiates glycosaminoglycan synthesis at the origin of the antithrombotic activity (Martin et al., 1996). Identification and characterization...
of the UGT isoforms able to glucuronidate the drug on the thioxyloside ring in humans and rat were achieved with several approaches: 1) use of metabolically competent, genetically engineered V79 cells expressing individual rat or human UGTs, 2) kinetics of the reaction and inhibitory effect of 7,7,7-triphenylheptanoic acid, a selective inhibitor of the glucuronidation reaction, and 3) study of the action of inducers on the glucuronidation of the drug in rat. The work will also define whether a single or distinct UGT isoform catalyzes the introduction of the glucuronic acid moiety on the different hydroxyl groups of LF 4.0212.

Materials and Methods

Chemicals. Uridine 5’-diphosphate-glucuronic acid (sodium salt) was purchased from Boehringer Mannheim (Mannheim, Germany). LF 4.0212 was a gift from Laboratoires Fournier (Daix, France). d-Saccharic acid 1,4-lactone (saccharonolactone), β-glucuronidase (bovine liver), digitonin, 3-methylcholanthrene, clofibrate, formic acid, and triethylamine were supplied by Sigma Chimie (St. Quentin Fallavier, France), and phenobarbital was supplied by Fluka (Buchs, Switzerland). β-Glucuronidase (Escherichia coli) was purchased from Sanofi Diagnostics Pasteur (Paris, France). Trifluoroacetic acid, trichloroacetic acid, glycine, and dimethyl sulfoxide (DMSO) were provided by Merck (Darmstadt, Germany) and acetonitrile was provided by BDH (Poole, UK). The inhibitor of the glucuronidation reaction, 7,7,7-triphenylheptanoic acid, was synthesized as described previously (Fournel-Gigleux et al., 1991). The 2-, 3-, and 4-O-glucuronide isomers of LF 4.0212 were synthesized and purified by Laboratoires Fournier. They were used as HPLC authentic standards. All other chemicals were of the best purity commercially available (or HPLC grade).

Liver Samples. Five human hepatic samples from transplantable livers were a gift from Professor E. Singlas and J. Valayer (Hôpital Kremlin-Bicêtre, Paris, France). Upon removal, the liver samples were kept in liquid nitrogen. The microsomes were prepared according to the method of Dragacci et al. (1987) and were suspended in 100 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol.

Male Wistar rats (180–200 g; Ifa Credo, St. Germain L’Abresle, France) were housed in an environmentally controlled room (12-h light/dark cycle, 22–24°C) and fed rodent chow (URA Alimentation, Villeneuvre, France). Rats were treated by the standard inducers, phenobarbital, 3-methylcholanthrene, or clofibrate, as described previously (Haumont et al., 1990). Liver microsomes were prepared according to the procedure of Hogeboom (1955) and were stored in 100 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. Protein content of microsomal fractions was determined by the technique of Bradford (1976) with bovine serum albumin as a standard.

Recombinant UGTs. Several recombinant V79 cell lines stably and individually expressing human (UGT1A6, UGT1A9, UGT2B4, and UGT2B11) and rat (UGT1A6 and 2B1) liver isoforms have been used to determine their potency to glucuronidate LF 4.0212. The UGT1A6 and 2B1 are 3-methylcholanthrene- and phenobarbital-inducible, respectively. Some of them (UGT1A6, UGT2B1, UGT2B4, and UGT2B11) have been established successfully in our laboratory (Fournel-Gigleux et al., 1991; Pritchard et al., 1994). The expression of each recombinant isoform was verified by immunoblots and in terms of specific activity with probe substrates, before testing LF 4.0212: planar and short phenols (1-naphthol, 4-methylumbelliferone) for the rat and human UGT1A6, chloramphenicol for UGT1A9, hyodeoxycholic acid for UGT2B4, 1-naphthol and 4-nitrophenol for UGT2B11, and morphine and ketoprofen for UGT2B1 (Pillot et al., 1993; Jin et al., 1993; Pritchard et al., 1994; Guengerich et al., 1997).

V79 Chinese hamster lung fibroblasts (donated by Dr. Marzin, Institut Pasteur, Lille, France) were grown in Dulbecco’s modified Eagle’s medium (Gibco, Cergy-Pontoise, France) without sodium pyruvate, supplemented with 4.5 g/liter glucose, 10% (v/v) Nu-serum (Collaborative Research Inc., Lexing-
ton, MA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Boehringer Mannheim). The cloning and stable expression of UGTs are detailed elsewhere (Fournel-Gigleux et al., 1991; Pritchard et al., 1994). Cells cultured for 72 h after replating were rinsed twice with ice-cold, phosphate-buffered saline, pH 7.4, harvested by scraping, and centrifuged for 15 min at 1000g (4°C). The resulting pellet was suspended in phosphate-buffered saline, centrifuged again (5000g, 5 min), and stored at −80°C until required. Cell homogenates were prepared by sononating the cellular pellets resuspended in 200 μl 100 mM Tris-HCl buffer (pH 7.4), 3 times for 5 s in ice, with a Vibra-Cell sonicator, 40 W (Biostrab, Strasbourg, France). Nontransfected V79 cells were used as positive controls.

Enzyme Assays. The conditions for glucuronidation of LF 4.0212 with the various sources of UGTs were optimized in terms of time, protein concentration, detergent concentration, divalent ions, and incubation pH. In rat liver microsomes only, the enzyme was activated by preincubating microsomes on ice for 20 min with Triton X-100 at a detergent/protein weight ratio of 0.8. This step was omitted for cellular membranes of V79 expressing the UGT isozymes because a mechanical activation was achieved by sonication used to break the cells. Standard incubations were performed in Eppendorf tubes containing 100 mM Tris-HCl buffer (pH 7.4) with 5.0 mM MgCl2 and 5.0 mM MnCl2, micromolar proteins (10–100 μg), LF 4.0212 (2 mM) dissolved in 5 μl DMSO, 5 mM D-saccharic acid 1,4-lactone, and 1 to 2 mM UDP-glucuronic acid in a total volume of 100 μl. The mixture was incubated at 37°C for 20 to 90 min and the reaction was terminated by placing the tube in ice and adding 10 μl 6 N HCl. The final concentration in HCl (0.54 N) did not affect the stability of the glucuronide formed. The tubes were centrifuged for 10 min at 2500g and an aliquot of the supernatant was analyzed by HPLC. All assays were done in triplicate. Control experiments were always run by omitting UDP-glucuronic acid.

Apparent kinetic parameters (Km, Vmax) were determined using linear least-squares regression analysis of double-reciprocal plots of initial velocity versus LF 4.0212 concentration; the concentration of UDP-glucuronic acid was kept constant at 1.0 mM.

The glucuronidation reaction was tested in the presence of 7,7,7-triphenylethanoic acid, which was found previously to be a potent inhibitor of the glucuronidation reactions (Fournel-Gigleux et al., 1989; Said et al., 1992). One min before addition of 2 mM LF 4.0212, the inhibitor (in DMSO) was added to the incubation mixture at the final concentration 0.10 mM. A control experiment was run without addition of the inhibitor.

Identification of the Glucuronic Acid Conjugate, β-Glucuronidases. Glucuronide formation was verified by the susceptibility of the products to hydrolysis by two β-glucuronidases. For this purpose, β-glucuronidase from bovine liver (200 μl, 1,200 U) dissolved in 200 mM sodium acetate buffer (pH 5.5) was added to 100 μl of the medium after 30 min incubation without D-saccharic acid 1,4-lactone. The reaction was conducted for an additional 4 h at 37°C; it was stopped by addition of 6 N HCl and the products were analyzed by HPLC. When the β-glucuronidase from E. coli (54 U) was used, the enzyme was dissolved in 200 mM sodium phosphate buffer (pH 6.5) and the mixture was incubated for 30 min at 50°C. Control assays were simultaneously run under the same conditions, but without β-glucuronidase to estimate the stability of the glucuronide under such conditions.

Electron spray liquid chromatography-mass spectrometry. The glucuronides biosynthesized by human and rat liver microsomes were further characterized by electron spray mass spectrometry. The glucuronides were separated on an HPLC system (see below). Mass spectrometry was carried out using a Fisons Instrument VG Platform (Micromass Ltd., Manchester, UK). Cone voltage was set at 20 V and the temperature source was 120°C. Data were collected in both positive and negative modes and reprocessed by means of Mass Lynx software (Micromass).

Chromatographic Conditions. The HPLC system consisted of a solvent delivery pump (Merck 655 A-11), an injection valve fitted with a 100 μl loop (Rheodyne, Cotati, CA), and a computing integrator D-2000 (Merck). Spectrophotometric detection was operated with a UV variable wavelength detector (LKB, Broma, Sweden) set at 280 nm. The analytical MN Nucleosil 120–5 C18 column (250 × 4.6 mm, 5 μm) was provided by Macherey-Nagel (Hoerdt, France). The mobile phase was composed of 20% (v/v) acetonitrile, 0.015% (v/v) formic acid, and 0.030% (v/v) triethylamine in water (apparent pH 3.5). The mobile phase was filtered through a 0.45-μm microfilter (Sartorius, Palaiseau, France) and used at a flow rate of 1.0 ml/min. The chromatograms were run at room temperature. Quantitation of LF 4.0212 glucuronide was performed by comparison with calibration curves established upon injection of increasing amounts of pure LF 4.0212. The glucuronide and the parent drug were found to present the same molar extinction coefficient. The response (peak area) was linear from 0.12 to 15 nmol LF 4.0212 injected. The detection limit was 0.02 nmol glucuronide under our experimental conditions.

The HPLC system coupled to the electrospray mass spectrometry consisted of a solvent delivery pump (model PU 980; Jacso, Prolabo, Fontenay/Bois, France), an injection valve (model 7725i, Rheodyne) fitted with a 20-μl loop, an analytical column (125 × 2 mm, i.d.) prepacked with LiChropher RP18 end-capped (5 μm; Merck), and a UV spectrometric detector (model UV 975; Jacso). A postcolumn tee with a low dead volume was used as a 1:5 splitter to reduce the eluent flow rate entering the mass spectrometry source. Mobile phase was an acetonitrile-water (15:85, v/v) mixture containing 0.015% (v/v) formic acid and 0.030% (v/v) triethylamine (pH 3.5); flow rate was 0.2 ml/min and the UV detection was operated at 280 nm.

Results

Structural Identification of LF 4.0212 Glucuronides and Regioselectivity of the Glucuronidation. In no case did we observe evidence of degradation of LF 4.0212 during the incubation. This was tested specifically by incubating this compound with microsomes from either human or rat liver, or from V79 cells for 90 min at 37°C under our standard incubation conditions, except that UDP-glucuronic acid was omitted.

The biosynthesis of LF 4.0212 glucuronides by membrane fractions from rat or human liver was characterized by several approaches: 1) absence of glucuronide when UDP-glucuronic acid was omitted, 2) comparison of retention times of glucuronide isomers with authentic standards, 3) hydrolysis of the glucuronide by two β-glucuronidases from E. coli and bovine liver, and 4) analysis by electrospray mass spectrometry.

Incubation of LF 4.0212 by rat and human liver microsomes. A typical chromatogram profile resulting from the incubation of the drug with liver microsomes of rats is shown in Fig. 1. Three glucuronides were formed at retention times 7.8, 8.1, and 12.9 min. (Fig. 1A). These peaks disappeared when incubations were run without UDP-glucuronic acid (Fig. 1B). They corresponded to the 2-O-, 4-O-, and 3-O-glucuronide isomers, respectively, by comparison of retention times of authentic glucuronides (peaks 2, 4, and 3). The chromatogram also indicated that the 3-O-glucuronide isomer was the main metabolite biosynthesized by the rat liver microsomes. All three glucuronides formed from rat liver microsomes were degraded by the E. coli and the bovine β-glucuronidase (not shown).

On the other hand, incubation of the drug with human liver microsomes led to the formation of a single product at a retention time 7.8 min, which corresponded to the 2-O-glucuronide. This metabolite was absent when UDP-glucuronic acid was omitted. In contrast to the rat, the human liver microsomes UGTs were unable to catalyze the glucuronidation on the 3- or the 4-hydroxy position of the drug.

Electrospray liquid chromatography-mass spectrometry. A pseudo-molecular ion [M-H]−corresponding to the most intense peak was observed for the 2-O-glucuronide in the negative mode at a m/z 458 (Fig. 2A). Similar spectra were obtained with the 3- and 4-O-glucuronide isomers (not shown). The peak at m/z 193 was tentatively assigned to glucuronic acid. By comparison, whatever the isomer considered, the positive mode led to a more complicated spectrum of the LF 4.0212 glucuronide, with two peaks of interest at m/z values 561 and 266, respectively, corresponding to an adduct between the
glucuronide and triethylamine and to the aglycone with a loss of a
water molecule (Fig. 2B).

**Kinetics of LF 4.0212 Glucuronidation and UGT Isoforms Cat-
alyzing the Reaction.** *Human hepatic microsomes.* The optimal con-
ditions for the biosynthesis of the 2-O-glucuronide isomer of LF
4.0212 with human liver microsomes were established before the
determination of the apparent kinetic constants. The biosynthesis of
the 2-O-glucuronide isomer was linear up to 60 min for a protein
concentration range up to 20 \( \mu \)g in the incubation medium. Glu-
curonidation had a broad pH optimum between 7 and 8. Maximal
formation required the presence of both 5 mM Mn\(^{2+}\) and 5 mM
Mg\(^{2+}\), compared with 70% maximal biosynthesis with 10 mM Mg\(^{2+}\)
alone or 25% only with no added divalent metal ion. Therefore, a
concentration of 5 mM Mn\(^{2+}\) and 5 mM Mg\(^{2+}\) and a pH 7.4 were
used for the subsequent experiments.

We determine the interindividual variations observed for the glu-
curonidation of the drug with liver microsomes from five different
patients. In all cases, only the 2-O-glucuronide isomer was detected.
The mean ± S.D. for the formation of the metabolite was 21.25 ±
3.04 nmol/min-\( \mu \)g, with a coefficient of variation of 14.3%, thus
indicating a low interindividual variation.

The apparent \( K_m \) and \( V_{max} \) toward LF 4.0212 for the formation of
the 2-O-glucuronide isomer were 1.2 mM and 36.0 nmol/min-\( \mu \)g
(Table 1). For the same reaction, the apparent \( K_m \) for the cosubstrate
UDP-glucuronic acid was 0.20 mM at a fixed concentration of 2 mM LF 4.0212 and the $V_{\text{max}}$ was 29.0 nmol/min/mg protein (data not shown).

Rat liver microsomes. For synthesis of all three products, the glucuronidation reaction was linear with respect to time of incubation (up to 60 min) and protein concentration (up to 0.100 mg). 10 mM Mg2+ or 10 mM Mn2+ were equally effective in stimulating the glucuronidation rate.

The specific activity of LF 4.0212 glucuronidation in rat was very low when compared with humans (Table 2). On the other hand, rat liver microsomes could glucuronidate on any one of the three different hydroxyl groups of the thioxyloxirane ring, although to different extents. The 3-O-glucuronide isomer was the major metabolite in rat (Table 2).

Interestingly, all of the UGT standard inducers considered in this study could markedly increase the glucuronidation rates on the three positions of the drug. Maximal increases were observed upon treatment with 3-methylcholanthrene and clofibrate for the formation of the 2-O-glucuronide isomer (Table 2).

Because of the very low activity observed with microsomes from untreated rats, the apparent kinetic constants $K_m$, $V_{\text{max}}$, and $V_{\text{max}}/K_m$ toward LF 4.0212 were expressed as mM, nmol/min·mg protein, and ml/mg/min·mg protein, respectively. Membrane fractions were incubated with increasing amounts of LF 4.0212 (0.01–0.50 mM), with a fixed UDP-glucuronic acid concentration (1 mM). Data for rat were obtained with animals treated with 3-methylcholanthrene and clofibrate for the formation of conjugates.

The 3- and 4-O-glucuronide isomers were equally effective in stimulating the glucuronidation of LF 4.0212 (Table 2). By contrast, the human UGT1A6 and UGT2B11 failed to glucuronidate the drug on this position. The rat liver recombinant UGT1A6 could also form 4-O-glucuronide and presented a specific activity similar to that obtained with the human orthologous form. The rat liver UGT2B1 was very active for the glucuronidation of LF 4.0212 on the 4-hydroxy position when compared with the other metabolic competent cell lines and even to the nontransfected V79 (Table 3). Finally, none of the cell lines tested could glucuronidate the drug on the 3-position.

The apparent kinetic constants $K_m$ and $V_{\text{max}}$ were measured for the most active recombinant cell lines as well as for the nontransfected cells (Table 2). Interestingly, the values for the formation of the 4-O-glucuronide isomer in the cells expressing the human liver recombinant UGT1A6 and UGT1A9 were very similar and not very different from those observed in nontransfected cells, thus suggesting that the biosynthesis of the 4-O-glucuronide isomer was catalyzed by an endogenous UGT. Finally, in terms of $K_m$, the affinity of UGT1A9 for glucuronidation on the 4-hydroxyl position was 27 times greater than for the 2 position (Table 1).

Table 3 indicates that the biosynthesis of the 3-O-glucuronide occurred with the highest efficiency in rat liver microsomes (Table 1).

Use of genetically engineered V79 expressing UGT cDNAs. Using microsomes from nontransfected V79 fibroblasts, the only product was the 4-O-glucuronide isomer of LF 4.0212 (Table 3). Its biosynthesis was linear for 60 min. It had a broad pH optimum, 7.5 to 8.8, and was highest with 10 mM MnCl2. Interestingly, membrane fractions from all available recombinant V79 cell constructs had similar specific activities for synthesis of the 4-O-glucuronide isomer, ranging from 0.11 to 0.28 nmol/min·mg, as shown in Table 3.

The 2-O-glucuronide isomer was biosynthesized by the human UGTs 1A9 and 2B4 (Table 3). By contrast, the human UGT1A6 and UGT2B11 failed to glucuronidate the drug on this position. The rat liver recombinant UGT1A6 could also form 4-O-glucuronide and presented a specific activity similar to that obtained with the human orthologous form. The rat liver UGT2B1 was very active for the glucuronidation of LF 4.0212 on the 4-hydroxy position when compared with the other metabolic competent cell lines and even to the nontransfected V79 (Table 3). Finally, none of the cell lines tested could glucuronidate the drug on the 3-position.

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Effect of triphenylheptanoic acid on glucuronidation of LF 4.0212. We previously reported that 7,7,7-triphenylheptanoic acid was an effective inhibitor of UGTs (Fournel-Gigleux et al., 1989; Said et al., 1992). This compound was therefore tested to determine if the glu-
The in vitro glucuronidation of LF 4.0212 was characterized in rat and humans. These two species presented radically different patterns of glucuronidation of the drug in terms of regioselectivity and metabolic rate. The 2-O-glucuronide isomer was the only metabolite found in humans, but rat could glucuronidate on all three positions, the main metabolite being the 3-O-glucuronide isomer. Additionally, human liver microsomes were much more active in glucuronidating LF 4.0212. The results indicate that the rat is not a suitable animal model to evaluate the glucuronidation of the drug in humans. This is also true for the glucuronidation of other carbohydrate-containing drugs, because we previously reported (Haumont et al., 1990) that rat glucuronidated AZT at a low rate compared to humans. These differences in the regioselectivity and in the efficiency of the glucuronidation process suggest that distinct UGT isoforms are involved in the glucuronidation of LF 4.0212 in rat and humans.

Surprisingly in humans, the glucuronidation on the thioxyloside ring proceeded with a very high rate when compared with that observed on the ribose moiety of AZT. We found (Haumont et al., 1990) that AZT glucuronidation had a $V_{\text{max}}$ (0.98 nmol/min/mg protein) 36-fold lower than that reported in this study for the formation of the 2-O-glucuronide isomer. LF 4.0212 was a better substrate than AZT ($V_{\text{max}}/K_{m}$ 30 and 13 µmol/min/mg, respectively). Compared with glucuronidation of AZT on the nonreactive 5’ hydroxyl of the ribose, the presence of the two electronegative sulfur atoms of the thioxyloside ring and of the thioglycoside bond could, by an attractive...
effect, increase the acidity of the vicinal 2-hydroxyl group, thus facilitating its glucuronidation. In that respect, the specific activity found in humans can be favorably compared with that reported for phenolic molecules, such as 1-naphthol and 4-methylumbelliferone, that are considered as good substrates of UGTs and are glucuronidated at high rates when compared with glucuronidation of carboxylic acids or amines (Magdalou et al., 1992; Huskey et al., 1994; Little et al., 1997).

Attempts to determine the isoform(s) involved in the glucuronidation of LF 4.0212 were performed by the aid of standard inducers of UGTs in rats and by genetically engineered V79-expressing single proteins. Phenobarbital, 3-methylcholanthrene, and clofibrate are substances known to stimulate the expression of distinct UGT isoforms belonging to families 1 and 2. 3-Methylcholanthrene induces preferentially the proteins of family 1 that glucuronidate planar phenols (1-naphthol, reduced naftozone; Okulicz-Kozaryn et al., 1981; Herber et al., 1995), whereas phenobarbital has a broader stimulating effect on the glucuronidation of bulky phenols, carboxylic acids such as nonsteroidal anti-inflammatory drugs (Magdalou et al., 1990), and aliphatic hydroxylated molecules catalyzed by isoforms of families 1 and 2. Finally, the peroxisome proliferator, clofibrate, induces specifically the bilirubin UGTs encoded by the UGT1 gene locus (Fournel et al., 1987; Magdalou et al., 1994). All these substances were powerful inducers, but exerted a nonspecific effect on LF 4.0212 glucuronidation, as the formation of all three position isomers was enhanced upon treatment. Interesting was the action of clofibrate, which strongly enhanced (11-times) the formation of the 2-O-glucuronide isomer. This hypolipidemic drug stimulates bilirubin glucuronidation, as the formation of all three position isomers was increased upon treatment. This suggests the action of clofibrate, which strongly enhanced (11-times) the formation of the 2-O-glucuronide isomer. However, part of this activity (0.283 nmol/min·mg protein) was due to the basal endogenous activity found with nontransfected V79 cells (see below). In comparison, V79 cells expressing the rat liver, 3-methylcholanthrene-inducible UGT1A6 was also active for glucuronidating the drug on the 4-hydroxy position with activity similar to that found in nontransfected cells.

The enzyme expressed in V79 cells did not catalyze the glucuronidation of LF 4.0212 on the 2- or 3-hydroxyl positions as found in rat liver microsomes. UGT2B1 is actively involved in the formation of both ether and ester glucuronides from aliphatic or aromatic origin, the best substrates being morphine or the nonsteroidal anti-inflammatory drugs of the series of 2-phenylpropionic acid (profens). The enzyme could glucuronidate AZT on the 5′ hydroxyl position, but at a very low rate (activity less than 0.01 nmol/min·mg protein; Pritchard et al., 1994).

Interestingly, the presence of an endogenous UGT able to form the 4-O-glucuronide isomer was detected in nontransfected V79 cell. The choice of V79 hamster lung fibroblasts as host cells for expression UGT cDNA was primarily dictated by the nondetectable or the very low glucuronidation activity at the level of the detection limit toward structurally unrelated xenobiotics. The nontransfected cells could actively glucuronidate LF 4.0212 on the 4-hydroxy position with apparent kinetic constants similar to those found in the genetically engineered cell lines. The apparent $K_m$, which was very low, and the $V_{max}$ values were similar in nontransfected V79 cells and in cells expressing UGT1A6 or UGT1A9. These data suggest that glucuronidation of the 4-hydroxyl of LF 4.0212 is catalyzed by UGT form(s) expressed in the host fibroblastic V79 cell line with a high affinity for the drug. UGT1A6 and UGT1A9 would not significantly contribute to the formation of 4-O-glucuronide isomer. This endogenous UGT exhibited distinct properties. No immunoreactive band was observed in nontransfected V79 cells using several types of antibodies raised against the full-length sequence of the rat liver UGT isoforms (Coughtrie et al., 1988) or against the N-terminal end of the human UGT1A6 and 2B4 (Fournel-Gigleux et al., 1991; Pritchard et al., 1994). Because it is not recognized by these probes directed against several domains of the protein, this UGT could be structurally different. The synthesis of LF 4.0212 4-O-glucuronide was not inhibited by 7,7,7-triphenylheptanoic acid, which has been found to be a very effective inhibitor of bilirubin and phenol glucuronidation in rat and human liver microsomes as well as of recombinant UGTs expressed in V79 cells (Said et al., 1992; Senay et al., 1997). Thus, the inability of this compound to inhibit the endogenous UGT also favors the existence of a different protein in nontransfected V79 cells. The same situation occurs in rat liver microsomes, for which 7,7,7-triphenylheptanoic acid could only partially inhibit the formation of 4-O-glucuronide. We believe that LF 4.0212, whose structure mimics the trisaccharidic moiety (xylose-galactose-galactose), could be used as a model substrate to study the class of UGTs that is involved in the biosynthesis of glycosaminoglycans. These enzymes are known to
incorporate glucuronic acid on such trisaccharides, leading to the formation of glycosidic polymers (Kitagawa et al., 1998).

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