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A Recombinant Phenobarbital-Inducible Rat Liver UDP-Glucuronosyltransferase (UDP-Glucuronosyltransferase 2B1) Stably Expressed in V79 Cells Catalyzes the Glucuronidation of Morphine, Phenols, and Carboxylic Acids

MIKE PRITCHARD,[1] SYLVIE FOURNEL-GIGLEUX, GÉRARD SIEST, PETER MACKENZIE, and JACQUES MAGDALOU

Centre du Médicament, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, 54000 Nancy, France (M.P., S.F.-G., G.S., J.M.), and Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, Australia (P.M.)

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SUMMARY

V79 (Chinese hamster lung fibroblast) cell lines expressing a functional recombinant phenobarbital-inducible rat liver UDP-glucuronosyltransferase (UGT), i.e., UGT2B1, were established. Western blot analysis of positive colonies, using anti-rat liver UGT antibodies, revealed the presence of an immunoreactive polypeptide of the expected molecular mass of 52 kDa. The substrate specificity of the recombinant enzyme toward >100 compounds was determined. Phenolic and alcoholic substrates included 4-methylumbelliferone, 4-hydroxybiphenyl, chloramphenicol, and testosterone, but a range of carboxylic acids of both endogenous (medium-chain saturated fatty acids, long-chain polyunsaturated fatty acids, and bile acids) and exogenous (profen nonsteroidal anti-inflammatory drugs, fibrate hypolipidemic agents, and sodium valproate) origin were also accepted, indicating that the enzyme was capable of forming both ether- and ester-type glucuronides from various structurally unrelated compounds. Determination of apparent kinetic constants for the glucuronidation by UGT2B1 of selected aglycones revealed a high maximal velocity toward the 3-position of morphine (49.3 ± 2.2 nmol/min/mg of protein), compared with other known substrates such as 4-methylumbelliferone (2.67 ± 0.11 nmol/min/mg of protein) or clofibric acid (0.06 ± 0.02 nmol/min/mg of protein). To gain a better insight into the mechanisms underlying the apparently wide substrate specificity of UGT2B1, series of structurally related compounds were tested as potential substrates. The rate of glucuronidation of unbranched saturated fatty acids and ω,ω,ω-triphenylalkanoic acids increased progressively with increasing alkyl chain length and then declined, with the best substrates in these two homologous series being decanoic acid and 4,4,4-triphenylbutanoic acid, respectively. Glucuronidation of *para*-substituted phenols always proceeded at a higher rate than that of the corresponding *para*-substituted benzoic acids. This could mean that the aglycon hydroxyl group was better positioned in the enzyme active site in the case of phenols. Alternatively, if the initial interaction with the enzyme required the aglycon to be in the protonated uncharged form, then the observation could be explained by the difference in ionization between phenols and benzoic acids at the incubation pH used. The introduction of a bulky alkyl group into the *para*-position led to increases of up to 300-fold in the rate of glucuronidation, probably as a result of the increased aglycon lipophilicity. Finally, the enzyme showed a degree of stereo- and regiospecificity, preferring (*S*)-ibuprofen to the *R*-enantiomer (V_{max}/K_m, 3.06 and 1.10 μl/min/mg of protein, respectively) and glucuronidating lithocholic acid but not hyodeoxycholic acid, which differs by only a single hydroxyl group. Given the wide substrate specificity of the recombinant enzyme, these transfected V79 cell lines may represent a useful *in vitro* model for investigating the molecular mechanisms of drug glucuronidation.

The UGTs (EC 2.4.1.17) are a multigenic family of membrane-bound enzymes, located mainly in the endoplasmic reticulum of liver and, to a lesser extent, in extrahepatic tissues such as kidney, small bowel, skin, and brain, that catalyze the transfer of a glucuronic acid moiety from the cofactor UDP-GlcA to a wide variety of exogenous and endogenous compounds containing a suitable hydroxyl, carboxyl, amino, or sulfhydryl group (1). The resulting glucuronides are more hydrophilic than the aglycons and can thus be more readily excreted from the body. Although glucuronidation is therefore usually considered a detoxifying pathway of drug metabolism, there are well documented examples of glucuronides that are pharmacologically more active than the aglycon (2), that are cholestatic (3), or that can form adducts with cellular proteins (4, 5), as is the case with carboxylic acid-containing drugs.

The instability of UGTs extracted from the endoplasmic

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[1] Present address: Biomedical Research Centre, University of Dundee, Ninewells Hospital and Medical School, Dundee, DD1 9SY, Scotland, UK.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDP-GlcA, UDP-glucuronic acid; TLC, thin layer chromatography; 4MU, 4-methylumbelliferone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DOPA, 3,4-dihydroxyphenylalanine.

reticulum by solubilization with detergents (1) has meant that the isolation, by conventional purification techniques, of individual isozymes in an active form and in sufficient amounts to allow detailed molecular characterization has proved to be difficult. Apart from a few exceptions such as bilirubin UGT (6, 7), this has been compounded by the lack of isozyme-specific substrates, making it almost impossible to distinguish between different isoforms in microsomal fractions or during purification. Nevertheless, a number of UGTs from different animal species have been purified to apparent homogeneity (8), allowing a limited characterization of certain isoenzymes in terms of molecular size, isoelectric point, substrate specificity, and glycosylation, as well as the production of several anti-UGT antibody preparations.

In the past few years, however, a number of cDNAs encoding complete UGT sequences have been isolated and cloned from tissues of rats, mice, and humans (1), which has led to the identification of two distinct families of UGTs, based on sequence comparison (9). Furthermore, these cDNAs have been expressed in cell lines by us and others, both transiently (10–12) and stably (13–15), allowing the recombinant UGTs to be screened for activity toward a limited number of endobiotic and mainly nontherapeutic xenobiotic substrates. Stable expression of individual isoforms of UGT in continuously dividing cells such as V79 hamster lung fibroblasts, which have low basal UGT activity, provides distinct advantages over other *in vitro* systems, because it allows the study of substrate specificity and kinetics without interference from other UGTs, using a reproducible and theoretically inexhaustible supply of material. It also allows the possibility of coexpressing several drug-metabolizing enzymes in the same cell.

In this paper, we report for the first time the stable expression of the cDNA UDPGT$_r$-2 (16–18), encoding the phenobarbital-inducible rat liver UGT2B1, in V79 cell lines. The apparently broad substrate specificity of this isoform may be of particular interest for the investigation of the molecular mechanisms of glucuronidation, especially of drugs whose glucuronides present important pharmacological or toxicological properties.

Materials and Methods

Radiochemicals. UDP-[U-^{14}C]-GlcA (10.55 GBq/mmol), [11,12-^{3}H(N)]retinoic acid (1.0 TBq/mmol), and [4-^{14}C]testosterone (1.9 GBq/mmol) were purchased from NEN (DuPont de Nemours, Dreiech, Germany), and α-[1-^{14}C]naphthol (0.26 GBq/mmol) was purchased from Sigma (St. Quentin Fallavier, France). The bile acids [^{3}H]hyodeoxycholic acid (2.10 GBq/mmol), 3α-hydroxy-5β-[^{3}H]androstane-17β-carboxylic acid (2.89 GBq/mmol), 3β-hydroxy-5β-[^{3}H]androstane-17β-carboxylic acid (1.73 GBq/mmol), and [^{14}C]lithocholic acid (1.81 GBq/mmol) were all kindly donated by Dr. A. Radominska (University of Arkansas Medical School, Little Rock, AR).

Drugs. Ibuprofen and flurbiprofen were kindly supplied by Boots-Dacour (Courbevoie, France), pirprofen by Ciba-Geigy (Rueil-Malmaison, France), ciprofibrate by Winthrop (Longvic, France), and lamotrigine and zidovudine by Wellcome (Paris, France). Sodium valproate was obtained from Labaz (Ambarès, France), furosemide from Hoechst (Puteaux, France), paracetamol from Bottu (Nanterre, France), lorazepam from Prophac (Paris, France), chloramphenicol from Fluka (Buchs, Switzerland), morphine hydrochloride from Cooperation Pharmaceutique Française (Melun, France), and morphine 3-glucuronide (92% purity) from Francopia (Paris, France). Bezafibrate, clofibric acid, diflunisal, indomethacin, ketoprofen, naproxen, oxazepam, probenecid, and 6-*n*-propyl-2-thiouracil were all purchased from Sigma, and salicylic acid and acetylsalicylic acid were obtained from Aldrich (St. Quentin Fallavier, France). The (*R*)- and (*S*)-ibuprofen enantiomers were purchased from Research Biochemicals Inc. (Natick, MA), and (*R*)- and (*S*)-ketoprofen were a kind gift of Dr. M. Abiteboul (Rhône-Poulenc-Rorer, Vitry-sur-Seine, France).

Other chemicals. UDP-GlcA (disodium salt) was obtained from Boehringer Mannheim (Meylan, France). The series of ω,ω,ω-triphenylalkanoic acids were synthesized and kindly donated by Drs. M. Said and J.-C. Ziegler (Université de Nancy I, France). Phenylacetic, diphenylacetic, and (*RS*)-2-phenylpropionic acids were purchased from EGA Chemie (Steinheim/Albuch, Germany), 5-hexenoic acid and 2,2- and 3,3-diphenylpropionic acids from Lancaster MTM Research Chemicals (Bischheim, France), and 2-naphthylamine from Prolabo (Paris, France). Lauric, myristic, palmitic, and stearic acids, 1- and 2-naphthylacetic acids, and 2-phenylbutyric acid were all purchased from Fluka, 4-methylphenol (*p*-cresol) from Janssen (Noisy le Grand, France), and L-DOPA from Calbiochem (Los Angeles, CA). The *R*- and *S*- enantiomers of 2-ethylhexanoic acid were supplied by Dr. H. Goudonnet (Université de Bourgogne, Dijon, France). All other chemicals were purchased from either Sigma, Aldrich, or Merck-Clevenot (Nogent-sur-Marne, France).

Cell culture and transfection. V79 Chinese hamster lung fibroblasts (kindly 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., Lexington, MA), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B (all from Boehringer Mannheim).

The cloning and transient expression of UDPGT$_r$-2 are detailed elsewhere (17). Co-transfection of the eukaryotic expression vector carrying the UDPGT$_r$-2 cDNA and a plasmid encoding the aminoglycoside resistance gene (SFVneo, kindly donated by Dr. H. Garoff, EMBL, Heidelberg, Germany) was carried out essentially as described previously (13). V79 cells were replated from early passage, 12 hr before transfection, at a seeding density of approximately 0.5 × 10^6 cells/90-mm plate, in 10 ml of medium. They were then incubated for 5 hr in 5 ml of serum-free medium containing plasmid UGT cDNA (15 μg), plasmid SFVneo DNA (2.25 μg), and Transfectam (IBF, Villeneuve-la-Garenne, France), used according to the manufacturer's recommendations. The incubation mixture was then replaced with normal medium, and cultures were split 1:6 after 24 hr. Selection medium, supplemented with G-418 sulfate (Geneticin, 1 mg/ml; GIBCO), was added 48 hr after transfection. After an additional 14 days, resistant colonies were either selected individually or pooled. These were then amplified and screened for UGT activity toward 4MU, a known substrate for the recombinant enzyme (17, 18), using the TLC method detailed below.

Cells cultured for 72 hr after replating were rinsed twice with ice-cold, sterile, phosphate-buffered saline, pH 7.4, harvested by scraping, and centrifuged at 4° for 15 min at 1000 × *g*. The resulting pellet was then resuspended in phosphate-buffered saline, recentrifuged (5000 × *g*, 5 min), and stored at −80° until required. UGT activities measured in cells stored in this way were stable for at least 9 months (data not shown).

Enzyme assays. Cell homogenates were prepared as described previously (13). All kinetic and substrate specificity determinations were performed on cells derived from one single colony and harvested at the same passage. The initial screening of the G-418-resistant V79 colonies and the subsequent determination of the substrate specificity of the recombinant UGT2B1 isoform were carried out using a general TLC method (20), modified as follows. Incubations were carried out at 37° for 30 min and consisted of 0.5 mM test substrate, added from a 10 mM stock solution in an appropriate solvent (usually methanol), 40–80 μg of cellular protein, 125 μM UDP-GlcA, 8.75 μM UDP-[U-^{14}C]-GlcA (230,000 dpm), and 10 mM $MgCl_2$ in 50 mM Tris·HCl buffer, pH 7.4, in a total volume of 40 μl. The test substrate concentration was reduced in incubations with bilirubin (0.1 mM), retinoic acid (0.25 mM), and the thyroid hormones (0.125 mM). Incubations with carboxylic acid substrates were carried out in 50 mM sodium phosphate buffer, pH 6.5, containing 5 mM D-saccharic acid 1,4-lactone (saccharolactone), to stabilize the acylglucuronides formed. Reactions were stopped by the

addition of 40 μl of ice-cold absolute ethanol. After centrifugation at 5000 × *g* for 5 min, 60 μl of supernatant were applied to a J.T. Baker Si250 PA (19C) silica gel TLC plate (Sochibo, Vélizy-Villacoublay, France), which was then developed in butan-1-ol/acetone/glacial acetic acid/30% ammonia/water (70:50:18:1.5:60, by volume). The plates were sprayed with a solution of 2-(biphenyl-4-yl)-5-(4-tert-butylphenyl)-1,3,4-oxadiazole (10 g/liter in toluene; Merck) before autoradiography. The scintillant used for quantification of radiolabeled glucuronides scraped from the plate was Fluoran Safe (BDH, Poole, England).

The glucuronidation of bile acids was also investigated by the method of Radominska-Pyrek *et al.* (21), using radiolabeled aglycon. Incubations consisted of 50 μM bile acid (prepared as mixed micelles with Brij 58; final concentration, 0.05%), 4 mM UDP-GlcA, 5 mM $MgCl_2$, 5 mM saccharolactone, and 50 μg of cell protein in 100 mM HEPES-NaOH buffer, pH 7.0, in a total volume of 60 μl. Incubations were carried out for 1 hr at 37° and then stopped with 20 μl of absolute ethanol. Separation of radiolabeled glucuronides and unreacted substrate was by TLC (see above), using a mobile phase of chloroform/methanol/acetic acid/water (65:25:2:4, by volume). Plates were sprayed with EN³HANCE (DuPont de Nemours) before autoradiography.

Glucuronidation of all-*trans*-retinoic acid was investigated using essentially the same method as that described above, except that incubations contained 100 μM substrate, 3 mM UDP-GlcA, and 100 μg of protein. Incubations with rat liver microsomes were run in parallel, to serve as positive controls. All procedures involving this substrate were carried out under yellow light to minimize photoisomerization.

Apparent kinetic parameters (K_m and V_{max}) for the glucuronidation of selected substrates of UGT2B1 were determined in V79 cell homogenates, using linear least-squares regression analysis of double-reciprocal plots of initial velocity versus substrate (or UDP-GlcA) concentration. The assays used were all based on published methods, with the substrates investigated being 4MU (22), morphine (23), clofibric acid, (*R*)-ibuprofen, and (*S*)-ibuprofen (24).

Protein concentrations were estimated by a modification of the method of Bradford (25), using bovine serum albumin (Sigma) as the standard.

Immunoblotting. Proteins from homogenates of V79 UDPGT$_r$-2 cells and control nontransfected V79 cells were separated by electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate, according to the method of Laemmli (26). They were then transferred onto nitrocellulose membranes and probed with polyclonal anti-rat liver testosterone/4-nitrophenol UGT antibodies [RAL1 (27), a kind gift of Pr. B. Burchell, Department of Biochemical Medicine, Ninewells Hospital and Medical School, Dundee, Scotland]. Anti-goat IgG alkaline phosphatase conjugates (Sigma) were used as secondary antibodies, and the recognized proteins were then visualized (28) using the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (GIBCO-BRL).

Results and Discussion

Expression of recombinant UGT2B1 in V79 cell lines. Thirty-one resistant colonies, with UGT activities toward 4MU ranging from 0 to 0.37 nmol/min/mg of protein, were isolated, of which one (C21) was selected for further investigation. These cells were amplified over three passages to provide a large stock of working material, while maintaining stable expression of the UGT cDNA (data not shown).

The establishment of these V79 cell lines provided a large and homogeneous source of functional protein, which allowed us to examine the specificity of UGT2B1 with respect to a wide range of potential substrates. In addition, intertransfection variability, inherent to the use of transient expression, could be avoided.

Immunoblotting of recombinant UGT2B1 expressed in V79 cells. Western blotting of homogenates of nontransfected V79 cells and V79 UDPGT$_r$-2 C21 cells with anti-rat liver UGT antibodies (Fig. 1) revealed the presence in transfected cells of a single immunoreactive polypeptide, of apparent molecular mass 52 kDa, that was absent in the nontransfected controls. This molecular mass was identical to that of the protein produced from the same cDNA in an *in vitro* transcription-translation system (17), suggesting that the foreign gene was correctly transcribed and translated in the V79 cells.

Examination of the substrate specificity of recombinant UGT2B1. Having established that the recombinant protein was expressed in V79 cells in sufficient amounts to be detected on immunoblot and that this protein was catalytically active, an extensive investigation into the substrate specificity of the recombinant enzyme was carried out. Cell homogenates were prepared in such a way as to remove the latency of the expressed UGT (29), thus ensuring the comparability of results obtained with different aglycons and allowing the determination of kinetic constants with the knowledge that the problem of substrate accessibility had effectively been eliminated.

Assay sensitivity was decreased for a number of aglycons, because the corresponding glucuronides had the same R_f value as radioactive products that were present in control incubations without substrate. Although no attempt was made during this study to characterize these endogenous products, the appearance of which has been described previously (18, 29), their formation was clearly pH dependent, being equivalent to an activity of 7.9 ± 1.8 pmol/min/mg of protein at pH 7.4, 88.4 ± 6.1 pmol/min/mg of protein at pH 7.0, and 17.4 ± 1.9 pmol/min/mg of protein at pH 6.5 (mean ± standard deviation, three

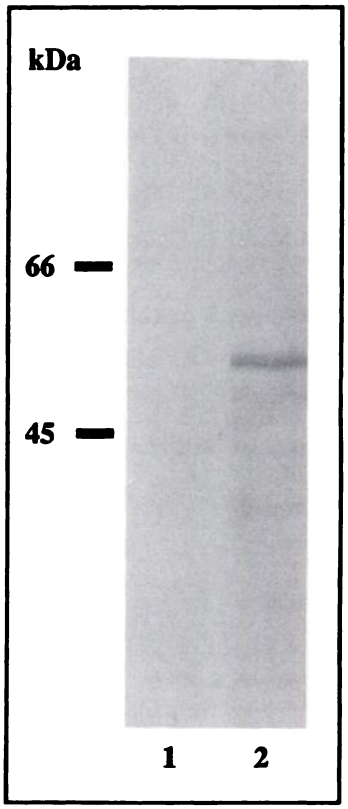

Fig. 1. Immunoblot analysis of recombinant UGT2B1 expressed in transformed V79 cell lines. Cell homogenate proteins (25 μg) were separated by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate, transferred to nitrocellulose membranes, and probed as described in Materials and Methods. *Lane 1*, control nontransfected V79 cells; *lane 2*, V79 cells expressing the UDPGT$_r$-2 cDNA. Molecular mass standards (kDa) are indicated on the *left*.

experiments), suggesting that they may have been acylglucuronides (30). In addition, both their R_f value and their apparent rates of formation were similar to those of the glucuronides of the longer-chain saturated fatty acids (data not shown), which are endogenous substrates for UGT2B1 (Fig. 2A). Attempts to reduce the background radioactivity (blank) resulting from this endogenous glucuronide formation by preincubation of V79 cell homogenates with unlabeled UDP-GlcA for up to 40 min before the addition of aglycon and UDP-[U-^{14}C]-GlcA were unsuccessful.

Xenobiotic substrates for UGT2B1. The results of the xenobiotic substrate screening of UGT2B1 are shown in Table 1. Due to the co-migration of the glucuronide(s) of morphine with unreacted UDP-GlcA in the TLC system used, screening for glucuronidation of this substrate was carried out using an alternative method (23), which had the added advantage of allowing formation of the 3-glucuronide (phenolic) and the 6-glucuronide (alcoholic) to be distinguished. The concentrations of aglycon and UDP-GlcA used were the same as those used in the TLC assay. Under these conditions, morphine was found to be glucuronidated by UGT2B1 with an activity of 2.55 nmol/min/mg of protein (the highest found during screening) and exclusively at the 3-position. This finding is in agreement with a study that showed that natural morphine was glucuronidated exclusively at the 3-position in rat liver microsomes (31).

Among the other xenobiotics tested (Table 1), the highest activities were found towards 4MU and 4-hydroxybiphenyl (two "classical" substrates of UGT) and 2-ethylhexanoic acid, a metabolite of the plasticizer 2-ethylhexylphthalate that is under investigation as a result of its peroxisome-proliferating effect

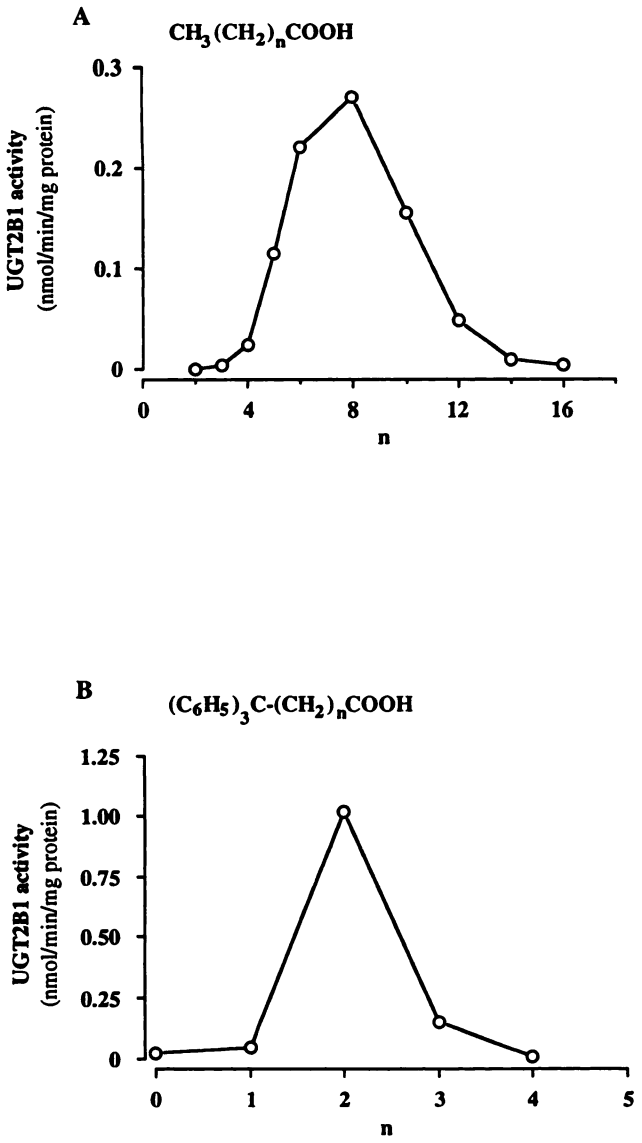

Fig. 2. Glucuronidation of carboxylic acids by recombinant UGT2B1 expressed in V79 cells. The activity of UGT2B1 toward two homologous series of carboxylic acids was investigated using a general TLC assay, as described in Materials and Methods. Activities are expressed in nmol of glucuronide formed/min/mg of cell homogenate protein and are the means of two determinations. *n*, number of methylene groups in the molecule. A, Unbranched saturated fatty acids; B, ω,ω,ω-triphenylalkanoic acids.

TABLE 1

Xenobiotic substrates of recombinant UGT2B1 expressed in V79 cell lines

Glucuronidation of the substrates (0.5 mM) was quantified by a general TLC assay, as described in Materials and Methods. Results are expressed as the mean of two determinations.

Xenobiotic	Activity
	nmol/min/mg of protein
Nonsteroidal anti-inflammatory drugs and chemically related substances	
(*R*)-Ibuprofen	0.06
(*S*)-Ibuprofen	0.22
(*RS*)-Ibuprofen	0.14
(*R*)-Ketoprofen	0.18
(*S*)-Ketoprofen	0.16
(*RS*)-Ketoprofen	0.16
(*S*)-Naproxen	0.07
(*RS*)-Pirprofen	0.14
(*RS*)-Flurbiprofen	0.12
(*R*)-(−)-2-Phenylpropionic acid	0.20
(*S*)-(+)-2-Phenylpropionic acid	0.15
(*RS*)-2-Phenylpropionic acid	0.30
Diflunisal	<0.01[a]
Salicylic acid	<0.01
Aspirin	<0.01
Paracetamol	ND[b]
Indomethacin	ND
Other drugs	
Chloramphenicol	0.28
Sodium valproate	0.14
Clofibric acid	0.05
Bezafibrate	0.02
Ciprofibrate	0.01
Zidovudine	0.01
Oxazepam	<0.01
Lorazepam	<0.01
Lamotrigine	ND
Probenecid	ND
Furosemide	ND
Phenolphthalein	ND
6-*n*-Propyl-2-thiouracil	ND
Other xenobiotics	
4MU	1.20
4-Hydroxybiphenyl	0.74
1-Naphthol	0.03
2-Naphthol	0.28
1-Naphthylacetic acid	0.30
2-Naphthylacetic acid	0.10
2-Naphthylamine	<0.01
Phthalic acid	<0.01
(*R*)-(−)-2-Ethylhexanoic acid	0.26
(*S*)-(+)-2-Ethylhexanoic acid	0.28
(*RS*)-2-Ethylhexanoic acid	0.40
(*RS*)-2-Ethylhexanol	0.50

[a] Limit of detection, 3 pmol/min/mg of protein.
[b] ND, not detected.

in the rat (32). Interestingly, the enzyme also showed activity towards another group of compounds known to be peroxisome proliferators, the fibrate hypolipidemic agents (33), indicating that UGT2B1 may play an important role in the metabolism of these compounds. No detectable activity was found toward either 6-*n*-propyl-2-thiouracil (thiol glucuronide) or the antiepileptic drug lamotrigine (quaternary ammonium-linked glucuronide). However, UGT activity toward lamotrigine in rat liver microsomes is known to be extremely low (34).

Because the level of the mRNA species encoding this particular isoform is increased by phenobarbital treatment, it was not surprising to find that the majority of the drug substrates found for UGT2B1 were those whose glucuronidation in rat liver is increased after treatment with this inducer (Table 1). Thus, besides morphine (31, 35) and clofibric acid (36), substrates included valproic acid (37), profen nonsteroidal anti-inflammatory drugs (38), (*S*)-naproxen (39), zidovudine (40), and chloramphenicol (35), which may explain why both clofibric acid and ibuprofen were competitive inhibitors of (*S*)-naproxen glucuronidation in liver microsomes isolated from phenobarbital-treated rats (39). Similarly, non-drug substrates included 4-hydroxybiphenyl (35) and 1- and 2-naphthylacetic acids (36).

One striking feature of the substrate screening was the number of carboxylic acid drugs that were accepted by UGT2B1. Indeed, comparison of activities obtained in this study with those determined previously in rat liver microsomes suggests that, relative to other substrates such as 1-naphthol and 4-methylumbelliferone, carboxylic acids represent one of the major classes of substrate for this enzyme. The only exceptions were those molecules in which the carboxyl moiety was adjacent to an aromatic carbon atom (for example, diflunisal and probenecid), which either were not substrates for UGT2B1 or were poor substrates. This has potentially important toxicological implications, because the formation of acylglucuronides has been associated with the appearance of protein adducts, which may, in turn, elicit an immune response (30, 41, 42). Certainly, the rates of glucuronidation of certain carboxylic acid drugs observed in this study were far greater than those determined for other recombinant UGTs (29, 43), which suggests that these cell lines may be a valuable tool for the investigation of the molecular mechanisms of acylglucuronide formation and toxicity.

The enzyme showed a degree of stereoselectivity, preferring (*S*)-ibuprofen to the *R*-enantiomer (see also Table 2), but demonstrated no apparent stereoselectivity for ketoprofen, 2-phenylpropionic acid, or 2-ethylhexanoic acid. A similar stereospecificity with regard to profens was recently observed for a recombinant human UGT from gene family 2 (43). Regiospecific differences were also observed, with the rate of glucuronidation of 2-naphthol being higher than that of 1-naphthol and, conversely, that of 1-naphthylacetic acid being higher than that of 2-naphthylacetic acid (Table 1). However, care must be taken when interpreting these results, because the K_m for 2-naphthylacetic acid in rat liver microsomes is relatively high, compared with that for 1-naphthylacetic acid (36). The regiospecificity of UGT2B1 toward xenobiotic substrates has been described previously (44), using the example of hydroxylated benzo[*a*]pyrenes. The enzyme preferentially glucuronidates the 1-, 2-, 8-, and 9-hydroxy metabolites and may thus play an important role in protecting the liver from the toxicity of these compounds.

Finally, UGT activities toward 4MU and clofibric acid were determined in a second, independently isolated, V79 UDPGT$_r$-2 colony (C18) and were found to be 1.00 and 0.05 nmol/min/mg of protein, respectively. The ratio of these activities is very similar to the ratio of the same activities determined in colony C21 (Table 1), thus providing indirect evidence that endogenous UGT genes that may have been activated during the transfection/amplification procedures were not contributing to the observed rates of glucuronidation.

Endobiotic substrates for UGT2B1. Among the endogenous compounds tested, the best substrates for UGT2B1 in terms of activity were the saturated fatty acids (Fig. 2A) (see below), particularly those of medium chain length, such as octanoic acid (activity, 195 ± 24 pmol/min/mg of protein; mean ± standard deviation, three experiments). Although the usual route of metabolism of such compounds is via β-oxidation to acetyl-CoA, octanoyl-β-D-glucuronide has been detected in the urine of patients with medium-chain acyl-CoA dehydrogenase deficiency (45) and would thus appear to have physiological significance. Indeed, it has been proposed to be a urinary marker for metabolic disorders of fatty acid metabolism (46). In contrast, decanoyl-β-D-glucuronide is not detectable in urine, and it has been suggested that this is due to the increased susceptibility of decanoic acid to ω-oxidation, compared with octanoic acid (46). It is interesting that UGT2B1 is able to glucuronidate some of the peroxisome proliferators that induce the enzyme(s) responsible for fatty acid ω-oxidation, as well as the fatty acids themselves.

The unsaturated fatty acids linoleic acid, α-linolenic acid, and arachidonic acid were also glucuronidated by UGT2B1, with activities of 0.07, 0.10, and 0.12 nmol/min/mg of protein, respectively. These activities were much lower than those toward long-chain saturated fatty acids (see Fig. 2A), perhaps as a result of the rotational restriction imparted by the double

TABLE 2

Apparent kinetic parameters for the glucuronidation of selected substrates by recombinant UGT2B1

UGT2B1 activities were determined using the assays described in Materials and Methods. Concentration ranges used for the aglycons were as follows: morphine, 0.5–20 mM; 4MU, 0.01–1 mM; clofibric acid, 0.01–0.2 mM; (*R*)-ibuprofen, 0.05–2 mM; (*S*)-ibuprofen, 0.02–1 mM. UDP-GlcA concentration was varied from 0.05 to 10 mM. Apparent kinetic constants were determined by double-reciprocal plot analysis. Results are expressed as mean ± standard deviation (three experiments).

Aglycons	V_{max}	K_m (aglycon)	K_m (UDP-GlcA)	V_{max}/K_m (aglycon)[a]
	nmol/min/mg of protein	*mM*	*mM*	*μl/min/mg of protein*
Morphine	49.3 ± 2.2	3.18 ± 0.38	0.22 ± 0.01	15.5
4MU	2.67 ± 0.11	0.130 ± 0.014	0.060 ± 0.011	20.5
Clofibric acid	0.055 ± 0.012	0.012 ± 0.001	0.058 ± 0.004	4.6
(*R*)-Ibuprofen	0.68 ± 0.02	0.62 ± 0.01	ND[b]	1.1
(*S*)-Ibuprofen	1.16 ± 0.02	0.38 ± 0.01	ND	3.1

[a] Means only.
[b] ND, not determined.

bonds. Activity (0.01 nmol/min/mg of protein) was also found toward prostaglandins E_1 and E_2, although it is unclear whether such glucuronides would be formed *in vivo* and, if so formed, what their physiological role would be. To our knowledge, the existence of these glucuronides has not been reported in the literature to date.

Among other endobiotics tested, low activity (0.01 nmol/min/mg of protein) was found toward homovanillic acid, but none was detected toward androsterone, estrone, all-*trans*-retinoic acid (see below), or serotonin. In addition, UGT2B1 was devoid of activity toward bilirubin. This finding is in agreement with the recent classification of UGT genes according to evolutionary divergence (9), which places all bilirubin UGTs identified to date in gene family 1, with family 2 being more involved in steroid and bile acid metabolism. It also supports previous studies in this laboratory that indicated that bilirubin was glucuronidated by UGT isoforms other than those that glucuronidated peroxisome proliferators (47).

Interestingly, UGT2B1 was found to glucuronidate L-thyroxine, with an activity of 18.8 ± 1.0 pmol/min/mg of protein (mean ± standard deviation, three experiments), but not 3,3′,5-triiodothyronine. This is supported by data that indicate that the two thyroid hormones are glucuronidated by different isoforms of UGT (48), and it also explains why the glucuronidation of thyroxine can be stimulated in rat liver by treatment with phenobarbital (49). The activity of UGT2B1 toward thyroxine obtained in this study, determined in nonfractionated cell homogenates, is much higher than values previously determined by other authors in rat liver microsomal fractions (slightly more than 1 pmol/min/mg of protein) (48–50), suggesting that the level of expression of the recombinant enzyme in the V79 cells is high. The fact that UGT2B1 was also able to glucuronidate L-DOPA (activity, 0.01 nmol/min/mg of protein), which resembles a part of the thyroxine molecule, but not dopamine, which lacks the carboxyl group, suggests that these products were carboxyl-linked glucuronides, although this obviously requires further investigation.

Finally, low activity (0.01 nmol/min/mg of protein) was found toward testosterone but no activity was seen toward 17β-estradiol, in contrast to results obtained when the same cDNA was expressed transiently in COS cells (18). However, using an assay based on radiolabeled steroid rather than radiolabeled UDP-GlcA, we have obtained evidence to suggest that activity toward testosterone was greatly underestimated by the TLC method, at least in part as a result of strong substrate inhibition at testosterone concentrations above 0.3 mM (data not shown). It is therefore quite possible that the enzyme also has activity toward 17β-estradiol but that an alternative assay is required to detect this. It should be borne in mind that, whereas the TLC method used in this study is invaluable for the rapid substrate screening of recombinant UGT, for a number of test compounds the incubation conditions used are far from optimal. Recent results obtained using recombinant UGT2B1 expressed in COS cells (51) indicate that the enzyme has distinct regioselectivity with regard to steroid substrates, preferring the 17β-hydroxyl group of C18 and C19 steroids.

The effect of several of the test compounds, including androsterone, lithocholic acid, and hyodeoxycholic acid, was to inhibit the endogenous glucuronide formation mentioned above, resulting in product signals that were less than the blank value and thus preventing the quantification of any aglycon glucuronide that may have been formed. For the bile acids, this problem was overcome by carrying out incubations with radiolabeled aglycon. UGT2B1 activities determined by this method (mean of two determinations) were 0.05 nmol/min/mg of protein with lithocholic acid, 0.06 nmol/min/mg of protein with 3α-hydroxy-5β-androstane-17β-carboxylic acid, and 0.12 nmol/min/mg of protein with 3β-hydroxy-5β-androstane-17β-carboxylic acid; the product in each case was an acylglucuronide. No detectable activity was found toward hyodeoxycholic acid (limit of detection, 1 pmol/min/mg of protein), which differs from lithocholic acid by a single hydroxyl group at the 6α-position. UGT2B1 thus showed distinct stereo- and regioselectivity with respect to bile acid substrates. Due to its increased sensitivity, this method was also used to test all-*trans*-retinoic acid as a substrate for UGT2B1, but no glucuronide formation was observed. This is in agreement with preliminary data obtained in our laboratory, which indicate that the glucuronidation of all-*trans*-retinoic acid in rat liver microsomes is inducible by 3-methylcholanthrene and clofibric acid but not by phenobarbital.[2]

Determination of kinetic parameters for glucuronidation by recombinant UGT2B1. Apparent kinetic parameters were determined for the glucuronidation by UGT2B1 of five substrates belonging to chemical classes (phenols and carboxylic acids) that would appear to represent major substrates of this enzyme. These results are shown in Table 2. The highest apparent V_{max} was that obtained for the 3-glucuronidation of morphine, i.e., almost 50 nmol/min/mg of protein. This activity compares favorably with the values of 34 ± 6 and 219 ± 42 nmol/min/mg of protein found previously in liver microsomes from untreated and phenobarbital-treated rats, respectively (31), suggesting again that the level of expression of UGT2B1 in the V79 cells was high.

In the past, it has often been difficult to correlate a recombinant UGT, derived from the heterologous expression of a cDNA in transfected cell lines, with a UGT purified or semi-purified from microsomal fractions. The xenobiotic substrate specificity of UGT2B1 suggests that it may correspond to (*R*)-naproxen GT_{pb}, described by El Mouelhi and Bock (39). Those authors used chromatofocusing to separate naproxen-glucuronidating activities in rat liver microsomes and isolated a peak with activity toward (*S*)-naproxen, morphine, 4-hydroxybiphenyl, and chloramphenicol that was selectively induced by phenobarbital treatment and that had an estimated isoelectric point of 7.8. The peak showed preferential enantioselectivity toward naproxen (S/R = 0.7). Unfortunately, (*R*)-naproxen was not available for us to confirm this finding. However, care must be taken when interpreting these results, because the chromatofocusing peak may have contained more than one isoform of UGT.

Although morphine seems to be its principal substrate, UGT2B1 is not the same enzyme as the phenobarbital-inducible morphine UGT that was purified previously from rat liver microsomes (52), because it differs in terms of molecular size, substrate specificity, and amino-terminal amino acid sequence (17, 52, 53). Similarly, it is not the same as the phenobarbital-inducible 4-hydroxybiphenyl UGT purified from rat liver microsomes (54), which was of similar molecular mass as UGT2B1 and glucuronidated similar substrates such as 4MU and 4-nitrophenol but which had no activity toward morphine or chloramphenicol and differed in the amino-terminal amino acid

[2] Unpublished observations.

sequence. However, these three proteins may well be closely related, as has been suggested recently (51), and they provide a good illustration of the overlapping but distinct substrate specificities of different isoforms of rat liver UGT.

In contrast to morphine, the apparent V_{max} obtained for clofibric acid was very low, approximately 50 pmol/min/mg of protein. However, in rat microsomes also, UGT activity toward clofibric acid is much lower than that toward morphine (24, 36). The apparent K_m (aglycon) was also much lower (by approximately 250-fold), suggesting that the affinity of UGT2B1 for this substrate could be higher than that for morphine. The apparent K_m (clofibric acid) found in this study (12 ± 1 μM) was identical to that found previously (11.5 ± 3.8 μM) for one of two sites determined for the glucuronidation of clofibric acid in liver microsomes isolated from phenobarbital-treated rats (24).

The stereoselectivity of the enzyme with respect to ibuprofen can be clearly seen, with the *S*-enantiomer being glucuronidated with both a higher apparent V_{max} and a lower apparent K_m, compared with the *R*-enantiomer, leading to an approximately 3-fold difference in capacity, as estimated by the V_{max}/K_m ratio. However, the highest ratio observed among these five substrates was for 4MU (Table 2). This was also the only aglycon that was glucuronidated at detectable levels in nontransfected V79 cell homogenates. These data fit a single-exponential kinetic function, with a V_{max} of 0.03 nmol/min/mg of protein and a K_m (4MU) of 202 μM.

Finally, it should be noted that for at least two of the five substrates the K_m (UDP-GlcA) was the same (Table 2). Logically, this should be the case, because we are dealing with the same isoenzyme and therefore the UDP-GlcA binding site should not change. The higher K_m (UDP-GlcA) seen with morphine as aglycon may reflect a slight deformation of the UDP-GlcA binding site and/or impaired UDP-GlcA binding due to the presence of the bulky morphine molecule.

Preliminary data suggest that the glucuronidation of certain other substrates (such as 1-naphthol, testosterone, and 1-naphthylacetic acid) by UGT2B1 in V79 cell homogenates follows two-site Michaelis-Menten kinetics. Experiments are underway to investigate this phenomenon further.

Glucuronidation of structurally related compounds by recombinant UGT2B1. To understand more clearly the molecular mechanisms of the reaction catalyzed by UGT2B1, an investigation into the glucuronidation by the enzyme of various series of structurally related compounds was carried out. Fig. 2 shows the results obtained with two homologous series of carboxylic acids, i.e., the unbranched saturated fatty acids (Fig. 2A) and the ω,ω,ω-triphenylalkanoic acids (Fig. 2B).

Among the saturated fatty acids, members of the group smaller than pentanoic (valeric) acid were not glucuronidated by UGT2B1, presumably due to their small size (insufficient hydrophobic interactions) and/or increasingly acidic nature. Introduction of more alkyl groups progressively increased the rate of glucuronidation, which reached a peak of 0.27 nmol/min/mg of protein with decanoic acid. The physiological significance of this medium-chain fatty acid glucuronidation has been discussed above. Increasing further the length of the alkyl chain led to increasingly poor substrates for UGT2B1, probably as a result of micelle formation but maybe also because of the increased number of rotational conformations of the molecule, reducing the possibility of finding a suitable fit with the binding site. Other compounds related to this homologous series were tested as substrates for UGT2B1. Of these, the results for 2-ethylhexanoic acid, an isomer of octanoic acid, have been already presented (Table 1). Glucuronidation of branched fatty acids, such as 2-ethylhexanoic acid and valproic acid (37), is more common, because their β-oxidation is impaired to some extent. Hexadecanedioic acid, a dicarboxylated intermediate in fatty acid metabolism, was glucuronidated at almost the same rate as hexadecanoic (palmitic) acid, and activity toward 5-hexenoic acid was about the same as that toward hexanoic acid. In contrast, no activity was detected toward pentadecafluorooctanoic acid, a derivative of octanoic acid used for industrial purposes that is known to produce peroxisome proliferation (33). Finally, the primary alcohols 1-hexanol and 1-octanol, despite having pK_a values greater than 15, were glucuronidated at only slightly lower rates than the corresponding hexanoic and octanoic acids (pK_a, 4.8–4.9), indicating that the glucuronidation mechanism is able to proceed even when it would appear to be difficult to remove the proton from the aglycon. Another example of this is shown in Table 1; 2-ethylhexanol was glucuronidated at a faster rate than the corresponding carboxylic acid.

The ω,ω,ω-triphenylalkanoic acids were originally synthesized as inhibitors of UGT activity toward bilirubin (55), with the most potent being 7,7,7-triphenylheptanoic acid (K_{iapp} = 5 μM). Interestingly, in this study, where the same compounds were being tested as substrates for a different UGT, 7,7,7-triphenylheptanoic acid was again inhibitory (as indeed was 7,7,7-triphenylheptanol), reducing the endogenous glucuronide formation almost to zero (data not shown). The other shorter members of the series were all glucuronidated to some degree by UGT2B1, with the fastest rate being found with 4,4,4-triphenylbutanoic acid (1.02 nmol/min/mg of protein). Indeed, the steep and narrow nature of the activity curve (Fig. 2B) suggests that this could be a good molecule with which to attempt to model the aglycon binding site of UGT2B1. We could perhaps envisage an aglycon binding site that consists of a hydrophobic pocket (into which the triphenyl moiety fits) and a site that interacts with the aglycon proton (see below), possibly by hydrogen bonding, separated by a distance roughly corresponding to that found in 4,4,4-triphenylbutanoic acid. Indeed, this may explain why the UGT2B1 activity is so much lower for the molecules differing from 4,4,4-triphenylbutanoic acid by just a single CH_2 group. Other contributory factors may include an increasing tendency towards UGT2B1 inhibition for the ω,ω,ω-triphenylpentanoic and -hexanoic acids and a rapid increase in acidity as the distance between the electron-withdrawing triphenyl moiety and the carboxyl group decreases (for example, addition of a single ω-phenyl group to butanoic acid decreases the pK_a from 4.81 to 4.76, whereas the same addition to acetic acid decreases the pK_a from 4.75 to 4.28).

The higher activities observed toward this second homologous series suggest that the additional hydrophobic interactions imparted by the triphenyl moiety may help in the fixation of the molecule to the aglycon binding site and may also reflect the reduced number of CH_2-CH_2 bonds, compared with the saturated fatty acids, which restricts the rotational freedom and thus increases the likelihood of finding a conformation to fit the aglycon binding/active site.

Experiments were also carried out using the first two members of the related homologous series of ω-phenylalkanoic and ω,ω-diphenylalkanoic acids. The rates of glucuronidation of phenyl-, diphenyl-, and triphenylacetic acids were 0.04, 0.23,

and 0.02 nmol/min/mg of protein, respectively. Corresponding values for the 3-phenyl-, 3,3-diphenyl-, and 3,3,3-triphenylpropionic acids were 0.10, 0.29, and 0.04 nmol/min/mg of protein, respectively. These results suggest that, of the three series, the triphenyl-substituted alkanoic acids are the poorest substrates for UGT2B1 and the diphenyl-substituted acids are the best. Unfortunately, longer members of the other two homologous series were not available for testing, although we might speculate that, of all these molecules, 4,4-diphenylbutanoic acid would be glucuronidated at the highest rate. Other related compounds found to be substrates for UGT2B1 were 2,2-diphenylpropionic acid (activity, 0.11 nmol/min/mg of protein) and 2-phenylbutanoic acid (0.24 nmol/min/mg of protein).

Glucuronidation of *para*-substituted phenols and benzoic acids by UGT2B1. Table 3 shows the results of the screening of UGT2B1 for activity toward a range of *para*-substituted phenols and benzoic acids. Glucuronidation of the phenols always proceeded at a faster rate than did that of the corresponding benzoic acids, suggesting either that the hydroxyl group was better positioned in the enzyme active site in the case of phenols or that the aglycon needs to be in the protonated uncharged form to interact with the aglycon binding site, in which case the data can be explained by the difference in ionization between phenols (pK_a, >9) and benzoic acids (pK_a, <5) at the incubation pH used.

Otherwise, there appeared to be little correlation between aglycon pK_a and the rate of glucuronidation by UGT2B1, because introduction into the phenol molecule of either an electron-withdrawing (such as bromo) or an electron-donating (such as methyl) *para*-substituent produced a large increase in activity. A similar effect was reported previously in a study of the glucuronidation of substituted phenols by rat liver microsomes (56); indeed, the relative UGT activities obtained in the two studies were remarkably similar. The pK_a of the aglycon hydroxyl or carboxyl group thus appears to be important only when it is considerably lower than the incubation pH, in which case the aglycon is predominantly in the deprotonated, negatively charged form and the rate of glucuronidation falls either as a direct result of this aglycon charge or as a result of the consequent decrease in aglycon lipophilicity (56).

TABLE 3

Glucuronidation of *para*-substituted phenols and benzoic acids by recombinant UGT2B1 expressed in V79 cell lines

Glucuronidation of the substrates was quantified by a general TLC assay, as described in Materials and Methods. Results are expressed as the mean of two determinations. pK_a values were obtained from the literature and refer to the aglycons in water at 25°.

para-Substituent	Phenols		Benzoic acids	
	pK_a	UGT2B1 activity	pK_a	UGT2B1 activity
		nmol/min/mg of protein		*nmol/min/mg of protein*
None	9.99	<0.005[a]	4.20	<0.005
Bromo	9.37	0.12	3.96	0.01
Nitro	7.15	0.15	3.43	ND[b]
Amino	10.3[c]	ND	4.87[d]	ND
Methoxy	10.21	0.05	4.50	0.02
Methyl	10.26	0.05	4.37	<0.005
Ethyl	10.2	0.38	4.35	0.07
Isopropyl	—[e]	0.61	4.40	NP[f]
t-Butyl	10.23	1.00	4.38	NP

[a] Limit of detection, 3 pmol/min/mg of protein.
[b] ND, not detected.
[c] pK_a (-NH_2), 5.48.
[d] pK_a (-NH_2), 2.50.
[e] —, Not available.
[f] NP, not performed.

A change in aglycon lipophilicity may also have contributed to the large increase in the rate of glucuronidation that resulted from the introduction of increasingly bulky alkyl substituents into the *para*- position (Table 3). Thus, UGT2B1 activity toward 4-phenylphenol (4-hydroxybiphenyl) (Table 1) was >200-fold higher than that toward phenol itself and that toward 4-*tert*-butylphenol was approximately 300-fold higher. Similar results were obtained for the glucuronidation of these compounds by another recombinant UGT, the human liver UGT 1*02 (UGT HP4) (14, 29). An effect of this magnitude could not have been due to a change in aglycon ionization, because this would have been very small at the incubation pH used. Instead, this observation may reflect improved access of the aglycon to the binding site, better orientation and/or fixation within this site, and/or a faster release of reaction products from the active site.

Other related compounds were also tested as substrates for UGT2B1. Activity toward 2- and 3-ethylphenol was much lower (0.02 and 0.05 nmol/min/mg of protein, respectively) than that toward 4-ethylphenol (Table 3), showing once again the regiospecificity of this enzyme. Benzyl alcohol was glucuronidated at about the same rate as phenol and benzoic acid (3 pmol/min/mg of protein), and phenylacetic acid was glucuronidated much more quickly (see above). However, the introduction of a *para*-methoxy group into either of these molecules had little or no effect on activity, in contrast to the increase observed as a result of the same introduction into phenol or benzoic acid (Table 3). Finally, 4-methylcyclohexanol was also glucuronidated by UGT2B1, but at a lower rate (0.01 nmol/min/mg of protein) than its aromatic counterpart 4-methylphenol.

Thus, UGT2B1 appears to catalyze the formation of both ether- and ester-type glucuronides of a wide range of structurally diverse compounds of both exogenous and endogenous origin, while at the same time showing distinct stereo- and regioselectivity. These transfected V79 cell lines may thus represent an invaluable *in vitro* model for the molecular characterization of an isoform of UGT that has not, to date, been purified to homogeneity. The wide substrate specificity of UGT2B1 suggests that the cell lines might also be useful for elucidating the molecular mechanisms of drug glucuronidation and, in particular, acylglucuronide formation and toxicity.

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Send reprint requests to: Jacques Magdalou, Centre du Médicament, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, 30 rue Lionnois, 54000 Nancy, France.