

Human and Rat Liver UDP-Glucuronosyltransferases Are Targets of Ketoprofen Acylglucuronide

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ABSTRACT

Acylglucuronides formed from carboxylic acids by UDP-glucuronosyltransferases (UGTs) are electrophilic metabolites able to covalently bind proteins. In this study, we demonstrate the reactivity of the acylglucuronide from the nonsteroidal anti-inflammatory drug, ketoprofen, toward human and rat liver UGTs. Ketoprofen acylglucuronide irreversibly inhibited the glucuronidation of 1-naphthol and 2-naphthol catalyzed by human liver microsomes or by the recombinant rat liver isoform, UGT2B1, which is the main isoform involved in the glucuronidation of the drug. A decrease of about 35% in the glucuronidation of 2-naphthol was observed when ketoprofen acylglucuronide was produced in situ in cultured V79 cells expressing UGT2B1. Inhibition was always associated with the formation of microsomal protein-ketoprofen adducts. The presence of these covalent adducts within the endoplasmic reticulum of cells expressing UGT2B1 was demonstrated following addition

of ketoprofen to culture medium by immunofluorescence microscopy with antiketoprofen antibodies. Immunoblots of liver microsomes incubated with ketoprofen acylglucuronide and probed with antiketoprofen antibodies revealed the presence of several protein adducts; among those was a major immunoreactive protein at 56 kDa, in the range of the apparent molecular mass of UGTs. The adduct formation partially prevented the photoincorporation of the UDP-glucuronic acid (UDP-GlcUA) analog, [β - 32 P]5N₃UDP-GlcUA, on the UGTs, suggesting that ketoprofen glucuronide covalently reacted with the UDP-GlcUA binding domain. Finally, UGT purification from rat liver microsomes incubated with ketoprofen glucuronide led to the isolation of UGT adducts recognized by both anti-UGT and antiketoprofen antibodies, providing strong evidence that UGTs are targets of this metabolite.

Glucuronidation is the major metabolic pathway for carboxylic acid containing drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) of the series of 2-phenylpropionic acid (profens), as well as diuretics and hypolipidemic agents. The reaction leads to the formation of acylglucuronides that are excreted in bile or urine. Unlike etherglucuronides formed from hydroxylated molecules, acylglucuronides are electrophilic species known to be intrinsically reactive both in vitro and in vivo (Spahn-Langguth and Benet, 1992). They undergo spontaneous hydrolysis to the parent drug as well as intramolecular rearrangement leading to β -glucuronidase-resistant 2-, 3-, and 4-*O*-acyl isomers. In addition, acylglucuronides bind covalently to endogenous macromolecules. Such irreversible binding with plasma pro-

teins has been reported by us and others for the acylglucuronides of several drugs including ketoprofen (Presle et al., 1996), tolmetin (Hyneck et al., 1988), zomepirac (Smith et al., 1990), ibuprofen and ibufenac (Castillo and Smith, 1995), and benoxaprofen (Spahn et al., 1990). It also has been documented that tissue proteins may be targets for acylation by metabolites of diflunisal (King and Dickinson, 1993), diclofenac (Kretz-Rommel and Boelsterli, 1994; Hargus et al., 1994), and tolmetin (Ojingwa et al., 1994). It has been postulated that proteins modified by the formation of adducts with drug acylglucuronides may cause immunological side effects and hepatotoxicity observed for these drugs (Olson et al., 1992). These effects have led to the withdrawal from the market of several NSAIDs, such as tolmetin, zomepirac, and benoxaprofen.

UDP-glucuronosyltransferases (UGTs) are a multigenic family of membrane-bound enzymes that are responsible for the glucuronidation of various drugs and endogenous com-

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; CHAPS, 3-[(3-chloroamidopropyl)dimethylammonio]-1-propane sulfonate; FITC, fluorescein isothiocyanate; PB, phenobarbital; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UDP-GlcUA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

pounds containing hydroxyl, carboxyl, amino, or sulfhydryl groups (Mackenzie et al., 1997). From the 50 UGT cDNA that have been isolated and characterized in rat and human until now, only two isoforms (UGT2B1 and UGT2B7) have been identified to glucuronidate NSAIDs chemically related to 2-phenylpropionic acid to an appreciable extent. The human UGT2B7 isoform, which has been expressed in HK293 cells, also glucuronidates catechol estrogens and androgens (Coffman et al., 1998). We recently stably expressed the cDNA encoding UGT2B1 in V79 fibroblasts (Pritchard et al., 1994). Analysis of the substrate specificity of this isoform revealed that carboxylic substances such as NSAIDs (ketoprofen, ibuprofen, and carprofen), hypolipidemic agents (clofibrate acid), and short-chain fatty acids were the major substrates of this enzyme, whereas hydroxylated substances, such as 2-naphthol, were also glucuronidated but at a lower rate. Because of its potency in catalyzing the formation of acylglucuronides, UGT2B1 is a model enzyme for studying the formation and reactivity of the acylglucuronides.

Ketoprofen, a widely used NSAID, is mainly glucuronidated in the liver as an acylglucuronide (Upton et al., 1980). Taking into account the reactivity of ketoprofen acylglucuronide and its main source of formation in the liver by the UGTs, it is likely that it could also bind to intracellular proteins, including UGTs themselves. In the present study, we investigated the reactivity of the glucuronide of racemic ketoprofen toward microsomal and recombinant UGT isoforms, particularly UGT2B1, responsible for the acylglucuronide formation. The results clearly show, for the first time, that ketoprofen glucuronide covalently binds to UGTs, which are irreversibly inactivated as a result of adduct formation.

Materials and Methods

Chemicals and Reagents

Ketoprofen [*R,S*-2-(3-benzoylphenyl)propionic acid], 1-naphthol, 2-naphthol, 1-naphthyl- β -D-glucuronide, D-saccharic acid 1,4-lactone, sodium cyanide, dimethyl sulfoxide, 3-[(3-chloroamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), paraformaldehyde, gelatin from bovine skin, and rabbit anti-goat and goat anti-rabbit alkaline phosphatase-conjugated IgG were obtained from Sigma (L'Isle d'Abeau, St. Quentin Fallavier, France). Saponin was provided by Aldrich (L'Isle d'Abeau, St. Quentin Fallavier, France). UDP-glucuronic acid (UDP-GlcUA) (sodium salt) was obtained from Boehringer (Mannheim, Germany). Dulbecco's modified Eagle's medium was obtained from Gibco-BRL (Eragny, France). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat and goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Affi-Gel Protein A mitogen-activated protein II was purchased from Bio-Rad (Ivry-sur-Seine, France). Diethylaminoethyl (DEAE)-Sephacel (DEAE-cellulose anion exchanger) was purchased from Sigma. Blue Sepharose CL-6B (Cibacron Blue 36-A covalently attached to Sepharose CL-6B by the triazine coupling method) was purchased from Pharmacia Biotech (St. Quentin-Yvelines, France). The UGT inhibitor, 7,7,7-triphenylheptanoic acid, was synthesized according to Fournel-Gigleux et al. (1989). The radiolabeled photoaffinity probe, [β - 32 P]5N₃UDP-GlcUA (specific activity 2–5 mCi/mmol), was synthesized as previously described (Drake et al., 1992).

Enzyme Fractions

Hepatic Microsomes. Human hepatic microsomes were prepared from transplantable livers according to the method of Dragacci et al. (1987). Male Wistar rats weighing 180 to 200 g were kept in an

environmentally controlled room (24°C, 12-h light cycle, constant humidity). For induction purpose, rats were given a single i.p. injection of phenobarbital (PB, sodium salt; Fluka, Buchs, Switzerland) at the dose of 100 mg/kg b.wt. (w/v) in NaCl 0.9%. PB was then added to the drinking water (1 g/liter) for 5 days. Liver microsomes from PB-treated rats were prepared by ultracentrifugation according to the method of Hogeboom (1955).

Membrane Fraction of Recombinant Cells Expressing UGT2B1 or UGT2B7. The establishment of the recombinant V79 cell lines (Chinese hamster lung fibroblasts) stably expressing the rat liver UGT2B1 and of the recombinant HK293 cell line stably expressing the human liver UGT2B7 has been described previously (Pritchard et al., 1994; Coffman et al., 1998).

Enriched membrane fractions of all recombinant cells were prepared from cell homogenates by differential ultracentrifugations, as previously described (Battaglia et al., 1994). Briefly, the cells were homogenized in 0.25 M sucrose, 5 mM HEPES (pH 7.4), and subjected to three 5-s sonications (Vibra Cell; Bioblock Scientific, Strasbourg, France). The homogenate was centrifuged at 10,000g for 10 min, and the resultant supernatant was centrifuged at 100,000g for 60 min. The final pellet, representing the enriched membrane fraction, was suspended in the same buffer and frozen at -80°C. The protein concentration was measured according to Lowry et al. (1951), with BSA (fraction V, Sigma) as standard.

Synthesis and Analysis of Ketoprofen Glucuronide

Ketoprofen glucuronide was prepared from PB-treated rat liver microsomes immobilized on alginate beads, as previously described (Haumont et al., 1991). Semipreparative HPLC was used to separate large amounts of biosynthesized glucuronide from UDP-GlcUA and ketoprofen. The HPLC apparatus consisted of a pump (L-6000 Hitachi; Merck, Darmstadt, Germany) equipped with an injector (Rheodyne valve with a 100- μ l sample loop; Rheodyne Inc., Cotati, CA). A 7- μ m Lichrosorb RP18 column (250 \times 10 mm; Merck, Darmstadt, Germany) was employed. The separated products were monitored by a UV detector (480 Waters Lambda Max; Milford, MA) coupled to a computing integrator (D-2000, Merck). The mobile phase consisted of acetonitrile/water/trifluoroacetic acid at a ratio 40:60:0.04 (v/v, pH 2.2) used at a flow rate of 2.5 ml/min. Detection of the glucuronide was performed at 256 nm. The eluted glucuronide was collected and dried under nitrogen. The mass and 1 H-NMR spectra of ketoprofen glucuronide were in accordance with the proposed structure. For analytical assays, HPLC separation of ketoprofen and ketoprofen glucuronide was performed on a 5- μ m Lichrospher RP18 column (125 \times 4 mm, Merck) with a mobile phase consisting of acetonitrile/water/trifluoroacetic acid at 35:65:0.04 (v/v, pH 2.2), at a flow rate of 0.8 ml/min. Quantitation of ketoprofen glucuronide was obtained from calibration curves drawn with ketoprofen, because the molar extinction coefficients at 256 nm of ketoprofen and ketoprofen glucuronide dissolved in the mobile phase are similar.

UGT Activity Measurement

1-Naphthol and 2-naphthol were used as reporter substrates for measurement of the glucuronidation activity supported by human hepatic microsomes and by membrane fractions of cells expressing UGT2B1.

The reaction mixture contained, in a total volume of 100 μ l, human hepatic microsomes (20 μ g protein) or membrane fractions of UGT2B1 (50 μ g protein) incubated in 100 mM Tris-HCl (pH 7.4) containing 10 mM MgCl₂ with 1 mM 1-naphthol or 2-naphthol dissolved in dimethyl sulfoxide at 37°C for 15 to 20 min. The reaction was initiated by the addition of 2 mM UDP-GlcUA. The reaction was stopped with 10 μ l 6 N HCl and the sample was centrifuged for 10 min at 5,000g.

HPLC quantitation of naphthyl- β -D-glucuronides was carried out on a 5- μ m Lichrospher RP18 column (125 \times 4 mm, Merck) with a mobile phase consisting of acetonitrile/water/trifluoroacetic acid (35:

65:0.04, v/v, pH 2.2). 1-Naphthyl- β -D-glucuronide was used as a standard. The flow rate was 0.5 ml/min and detection of glucuronides was performed at 229 nm. Specific activities of enzymes are expressed as nmol/min \cdot mg protein. Where applicable, means \pm S.D. are reported.

In Vitro Irreversible Binding of Ketoprofen Glucuronide to Proteins

For the analysis of irreversible inhibition, various concentrations of ketoprofen glucuronide (0–5 mM) were incubated with human hepatic microsomes or with membrane fractions of cells expressing UGT2B1 for various time periods (5–30 min) at 25°C in 400 μ l of 100 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, containing 5 mM D-saccharic acid 1,4-lactone, an inhibitor of β -glucuronidase. We verified that under these conditions, no significant hydrolysis of ketoprofen glucuronide to the parent drug occurred during the experiment. After centrifugation at 100,000g for 30 min, the protein pellet was washed two times with 200 μ l 100 mM Tris-HCl (pH 7.4), 10 mM MgCl₂ to remove unbound ketoprofen glucuronide and then homogenized in 350 μ l of the same buffer. We checked that washings reduced ketoprofen glucuronide levels to noninhibitory concentrations in the assays. Glucuronidation activity toward 1-naphthol or 2-naphthol was assayed as described above. A control experiment in which ketoprofen glucuronide was omitted was performed simultaneously and represented 100% enzyme activity.

To evaluate the amount of protein adducts, 30- μ l aliquots of washed proteins were treated with 500 μ l of 2 M NaOH at 65°C for 4 to 12 h. After acidification (200 μ l of HCl 6 N) and extraction of the protein digest with ethyl acetate (2 ml), the amount of ketoprofen released was quantitated by HPLC and corresponded to the irreversibly bound fraction. The concentration of covalently bound ketoprofen glucuronide was calculated as nanomoles of ketoprofen per milligram of total microsomal proteins present in the aliquot.

Analysis of Protein Adducts by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot

PB-treated rat liver microsomes were preincubated for 4 h at 25°C with 2.5 mM ketoprofen glucuronide in 100 μ l of 100 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, containing 5 mM D-saccharic acid 1,4-lactone, with 7 mM sodium cyanide. Proteins were precipitated by addition of 20 μ l of trichloroacetic acid 10% (w/v) and were centrifuged at 13,000g for 10 min. The protein pellet was washed with 500 μ l of methanol/ether 3:1 (v/v) and solubilized in sample loading buffer [125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, and 0.1% (w/v) bromophenol blue]. Aliquots were stored at –20°C until they were subjected to SDS-PAGE and Western blot analysis.

SDS-PAGE was performed according to Laemmli (1970), using a 4% (w/v) stacking gel and a 10% (w/v) running gel, with 100 μ g of proteins loaded per well. After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) by electroblotting. Immunodetection was accomplished using antiketoprofen polyclonal antibody obtained from female New Zealand White rabbits immunized against ketoprofen-thyreoglobulin by the method described for the production of anticarprofen antibodies (Maire-Gauthier et al., 1998). The antiketoprofen IgG were purified from rabbit plasma with a Affi-Gel Protein A antigen-activated protein II column (Bio-Rad) and were dialyzed overnight against a 0.1 M bicarbonate, 0.5 M NaCl buffer (pH 8.3) at 4°C. The antibody was able to detect as little as 4 pmol of ketoprofen. Antiketoprofen antibodies were diluted 1:3000 (v/v) and the secondary goat anti-rabbit IgG (alkaline phosphatase conjugate) were diluted 1:5000 (v/v).

In Situ Irreversible Binding of Ketoprofen Glucuronide to Proteins

UGT2B1-transfected V79 cells were grown to subconfluency in 90-mm diameter Petri dishes as described above. One day before the

experiment, 5 mM glucose (as a precursor of UDP-GlcUA) and 2 mM *n*-butyric acid (sodium salt) were added to the culture medium. Cells were then treated with 0.5% dimethyl sulfoxide (control) or with 1 mM ketoprofen dissolved in dimethyl sulfoxide [0.5% (v/v)] for 24 h at 37°C, in presence or absence of 2 mM 7,7,7-triphenylheptanoic acid dissolved in dimethyl sulfoxide [0.5% (v/v)]. At this point, samples were taken from the extracellular medium, acidified by HCl 6 N, centrifuged at 5000g for 10 min, and ketoprofen glucuronide content was evaluated by HPLC. The remaining culture medium was discarded and the plates were washed three times with the culture medium. Naphthol glucuronidation assays were then carried out in the same culture medium; the reaction was started by adding substrate [1 mM 2-naphthol dissolved in dimethyl sulfoxide, 0.5% (v/v)] to the medium and samples were incubated for 10 h at 37°C. Aliquots were taken from the extracellular medium after 10 h and total glucuronide formation and the amount of irreversible bound ketoprofen glucuronide to the cell proteins were determined as described above.

Immunofluorescence Microscopy

Control and UGT2B1-transfected V79 cells were grown and treated with ketoprofen as described above. The culture medium was discarded and the plates were washed three times with PBS. Cells were fixed with 2% (w/v) paraformaldehyde in PBS for 20 min, permeabilized with 0.2% (w/v) saponin in PBS for 40 min, and saturated with 0.2% (w/v) gelatin in PBS for 30 min. Two primary antibodies were used for immunodetection: anti-rat liver UGT antibody, which has been shown to react with different UGT isoforms (Coughtrie et al., 1988), and antiketoprofen antibody. Cells were incubated for 1 h with primary anti-UGT or antiketoprofen antibodies, at dilutions of 1:1000 (v/v) and 1:500 (v/v), respectively, in PBS containing 0.1% (w/v) saponin and 1.5% (w/v) BSA. Immunoreactive proteins were then visualized with FITC-conjugated donkey anti-goat IgG or goat anti-rabbit IgG at a dilution of 1:100 (v/v) for 30 min in PBS saline containing 0.1% (w/v) saponin and 1.5% (w/v) BSA. Fluorescence microscopy was performed using a Nikon UFX-DX microscope. Kodak Ektachrome 400 film was used for photography.

Purification of UGT Modified by Ketoprofen Adducts

UGT purification was performed according to the method of Coffman et al. (1996). PB-treated rat liver microsomes were incubated for 4 h at 25°C with 5 mM ketoprofen glucuronide in 8 ml of 100 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, containing 5 mM D-saccharic acid 1,4-lactone and 5 mM sodium cyanide. The microsomes containing the protein adducts (10 mg of protein) were solubilized at 4°C for 30 min by addition of CHAPS to a final concentration of 1 mg detergent/mg protein. The pH of the mixture was adjusted to pH 9.1 with Tris. The mixture was centrifuged at 100,000g for 30 min and the supernatant containing the solubilized material was stored at –20°C until the purification was performed.

The solubilized microsomes were loaded onto a DEAE-Sephacel column (Sigma; 5 cm, 1 cm diameter) equilibrated at 4°C with 50 ml of 25 mM Tris-acetate buffer (pH 9.1), 0.1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol, and 175 μ g/ml azolectin (Sigma). After loading the solubilized proteins, the column was washed with 20 ml of equilibration buffer and eluted with equilibration buffer containing 50 mM KCl until the absorbance (280 nm) was nearly 0. The UGT proteins were then eluted with 100 mM KCl. Fractions of 2.4 ml were collected and aliquots were subjected to Western blot analysis, as described above, with either anti-UGT or antiketoprofen antibodies. Fractions containing immunoreactive protein were dialyzed overnight at 4°C against 25 mM Tris-acetate buffer (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol, and 1% (w/v) CHAPS, and then applied to a Blue Sepharose CL-6B column (Pharmacia Biotech; 5 cm, 1 cm diameter) equilibrated with the same buffer. The column was washed with 2 column volumes of equilibrating buffer and was eluted with a 50-ml gradient ranging from 0 to 0.5 M NaCl.

Fractions of 2.4 ml were collected and aliquots were subjected to Western blot analysis with either anti-UGT or antiketoprofen antibodies.

Photoaffinity Labeling with [β - 32 P]5N $_3$ UDP-GlcUA

Human and rat liver microsomes and membrane fractions of UGT2B7-transfected HK293 cells were preincubated for 30 min at 25°C with 2.5 and 5 mM ketoprofen glucuronide, respectively, in 100 μ l of 100 mM Tris-HCl (pH 7.4), 10 mM MgCl $_2$, containing 5 mM D-saccharic acid 1,4-lactone. The inactivation was stopped by a 25-fold dilution with ice-cold 1 mM HEPES, pH 7.4, containing 0.25 M sucrose. The diluted microsomal proteins were then centrifuged at 100,000g for 30 min at 4°C. Pellets were resuspended in a minimal volume of 180 mM Tris-HCl buffer (pH 7.4) containing 28 mM MgCl $_2$, and the proteins were photolabeled as follows: microsomal proteins (about 50 μ g) were incubated with [β - 32 P]5N $_3$ UDP-GlcUA (40 μ M) in 180 mM Tris-HCl buffer (pH 7.4) containing 28 mM MgCl $_2$ for 1 min on ice, followed by an irradiation for 90 s with a handheld shortwave (254 nm) UV lamp (UVG-11, Ultraviolet Products, San Gabriel, CA). Proteins were processed for SDS-PAGE and run on a 10% (w/v) gel as previously described (Drake et al., 1992). After electrophoresis, the gels were dried and radiolabeled proteins were visualized by autoradiography for 5 days at -80°C. The percentage of photoincorporation was determined by densitometry using an imaging densitometer (IS-1000 Digital Imaging System, Alpha Innotech Corporation, San Leandro, CA).

Results

Irreversible Inhibition of UGT Activity by Ketoprofen Glucuronide. The covalent binding of ketoprofen glucuronide (0–5 mM) to human hepatic microsomes and to membrane fractions of UGT2B1-transfected V79 cells was evaluated after washings of the modified proteins. Simultaneously, the effect of ketoprofen glucuronide covalent binding on the glucuronidation capacity of the modified proteins was also investigated. The results as shown in Fig. 1, showed that the concentration-dependent inhibition of naphthol glucuronidation in human hepatic microsomes (Fig. 1A) or UGT2B1-V79 membranes (Fig. 1B) was strongly correlated to an increase of the amount of total ketoprofen adducts, suggesting that binding of ketoprofen glucuronide to proteins, including UGTs, led to inactivation of the modified proteins. Washings did not remove all the unbound ketoprofen glucuronide, but the residual concentration (0.6 mM in human hepatic microsomes treated with 4.5 mM ketoprofen glucuronide and 0.05 mM in membrane fractions of UGT2B1-V79 cells treated with 1.5 mM ketoprofen glucuronide) was too low to be responsible for the observed inhibition of the UGT enzymes (results not shown). To further investigate the mechanism of the ketoprofen glucuronide inhibition, we pre-incubated human hepatic microsomes (Fig. 2A) and UGT2B1-V79 membrane fractions (Fig. 2B) with increasing ketoprofen glucuronide concentrations for different periods of time. Ketoprofen glucuronide caused a time- and concentration-dependent loss of glucuronidation activity toward 1- and 2-naphthol, indicating an irreversible inhibition caused by the acylglucuronide. A large increase in UGT2B1 inhibition was observed when acylglucuronide concentration changed from 0.91 to 1.71 mM (Fig. 1B). The analysis of the plots revealed the existence of a mixed-type irreversible inhibition.

Characterization and Identification of Ketoprofen-Protein Adducts. The results presented above suggested that the inhibition of the glucuronidation reaction occurred

via covalent binding. To further identify the protein targets of ketoprofen glucuronide, several approaches were used: 1) the microsomal proteins were analyzed by Western blot using antiketoprofen antibodies; 2) the UGT adducts were purified and analyzed by Western blot; and 3) a photoaffinity probe analog of UDP-GlcUA known to bind covalently to the cosubstrate binding site (Drake et al., 1992) was used in an attempt to determine the domain on the UGT protein with which the ketoprofen glucuronide interacts.

PB-treated rat liver microsomes were incubated for 4 h with 2.5 mM ketoprofen glucuronide. Proteins were analyzed by SDS-PAGE and immunoblotted using antiketoprofen antibodies. When PB-treated rat liver microsomes were not treated with ketoprofen acylglucuronide, Western blot anal-

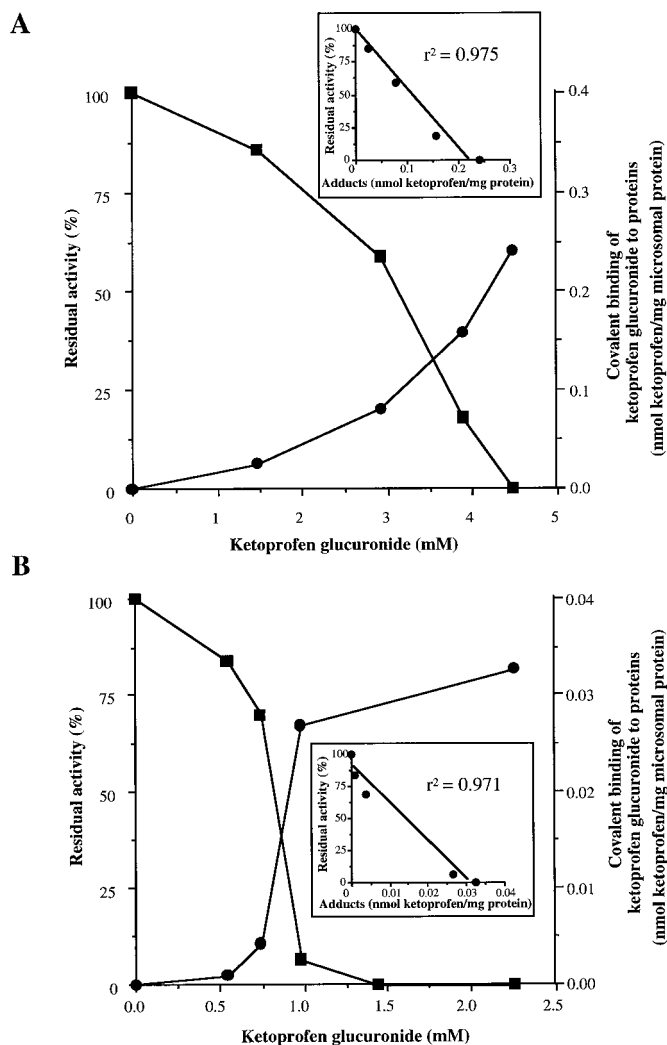


Fig. 1. Relationship between inhibition of 1- and 2-naphthol glucuronidation and covalent binding of ketoprofen glucuronide to proteins. A, inhibition was carried out with human hepatic microsomes in 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl $_2$ at 25°C, using various concentrations of ketoprofen glucuronide. Glucuronidation reaction was evaluated using 1-naphthol as substrate. B, inhibition was carried out with membrane fractions of UGT2B1-transfected V79 cells in 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl $_2$ at 25°C, using various concentrations of ketoprofen glucuronide. Glucuronidation reaction was evaluated using 2-naphthol as substrate. Inhibitions were quenched at 30 min. Residual activities (■) and amount of irreversibly bound glucuronide (●) were determined as described in *Materials and Methods*. Insets show the correlation between enzyme inactivation and total adduct formation.

ysis using antiketoprofen antibodies clearly showed no visible proteins (Fig. 3, lane 1). In contrast, several immunoreactive proteins were revealed by antiketoprofen antibodies when PB-treated rat liver microsomes were incubated with ketoprofen glucuronide. Moreover, the presence of a major immunoreactive band was demonstrated (Fig. 3, lane 2). The apparent molecular mass of this polypeptide was about 56 kDa, which was in the range of the molecular weight of UGTs (Coughtrie et al., 1988). The effect of ketoprofen glucuronide concentration on the intensity of the immunoreactive signals was examined by exposing PB-treated rat liver microsomes to increasing concentrations of ketoprofen glucuronide (0–4 mM) for 4 h. The immunoblot showed a concentration depen-

dence of the intensity of the band corresponding to the 56-kDa protein (results not shown).

To investigate further whether UGTs were indeed targets of ketoprofen glucuronide, purification by DEAE ion-exchange chromatography was carried out with solubilized PB-treated rat liver microsomes containing the protein adducts. SDS-PAGE analysis followed by Coomassie blue staining (Fig. 4A) showed a major polypeptide in the final purified material with an apparent molecular mass of 56 kDa, which immunoreacted with both anti-UGT and antiketoprofen antibodies (Fig. 4, B and C, lanes 4, 5, and 6). Immunoblot analysis of purified UGTs from nontreated rat liver microsomes, performed as control, showed no signal revealed by antiketoprofen antibodies. Interestingly, by contrast to unmodified UGTs, UGTs incubated with ketoprofen glucuronide failed to bind on a Blue Sepharose CL-6B column, used as a final affinity chromatography step, suggesting an important change in protein structure or conformation upon interaction with the metabolite (data not shown).

Photoaffinity experiments with [β - 32 P]5N₃UDP-GlcUA were performed with membrane fractions of UGT2B7-transfected HK293 cells (Fig. 5) preincubated with 0 (control) or 5 mM ketoprofen glucuronide. The inhibitory potency of 5 mM ketoprofen glucuronide on the glucuronidation of androstosterone catalyzed by UGT2B7 was about 50%. The analysis of the autoradiograms of the photolabeled proteins preincubated with 5 mM ketoprofen glucuronide showed a significant decrease (about 30%) of photoincorporation of the probe into UGT2B7 protein. Similar results were obtained when human or rat liver microsomes were photolabeled with the probe (results not shown). These data suggest that the ketoprofen adducts, which partially prevented the photolabeling of the UGTs, would compete with the probe for the UDP-GlcUA binding site, resulting in UGT inhibition.

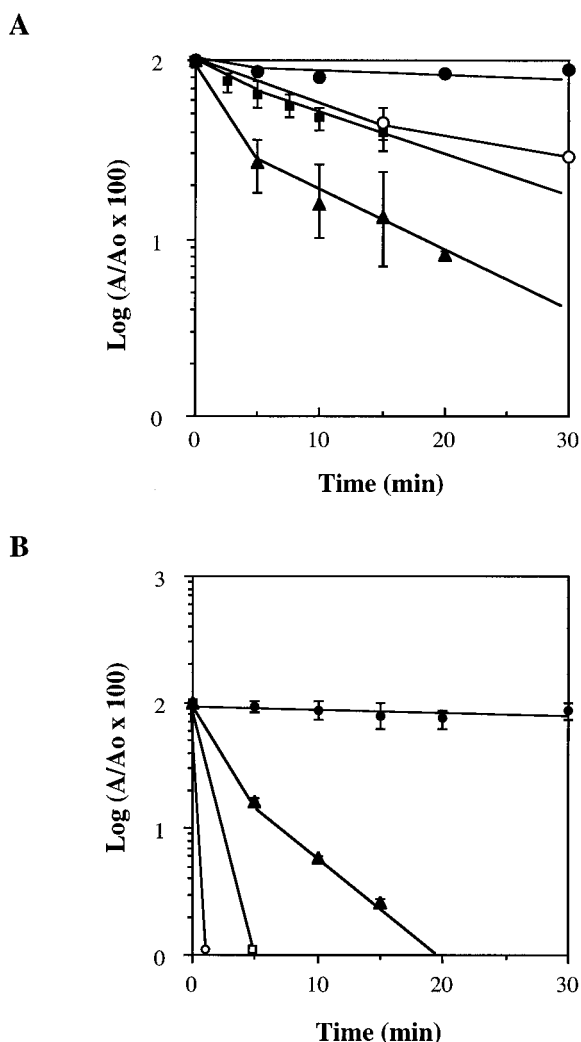


Fig. 2. Time-dependent inhibition of human hepatic microsomal UGTs and recombinant rat liver UGT2B1 by ketoprofen glucuronide. A, inhibition was carried out by incubating various concentrations of ketoprofen glucuronide (●, 1.47 mM; ○, 3.45 mM; ■, 3.65 mM; ▲, 3.91 mM) with human hepatic microsomes (600 μ g) in 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ at 25°C. Glucuronidation reaction was evaluated using 1-naphthol as substrate. B, inhibition was carried out by incubating various concentrations of ketoprofen glucuronide (●, 0.91 mM; ▲, 1.71 mM; □, 2.37 mM; ○, 2.71 mM) with membrane fractions of UGT2B1-transfected V79 cells (770 μ g) in 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂ at 25°C. Glucuronidation reaction was evaluated using 2-naphthol as substrate. Inactivations were quenched at the indicated times and the residual activity, A/Ao, was determined, as described in *Materials and Methods*. Each data point is the mean \pm S.D. of three separate determinations.

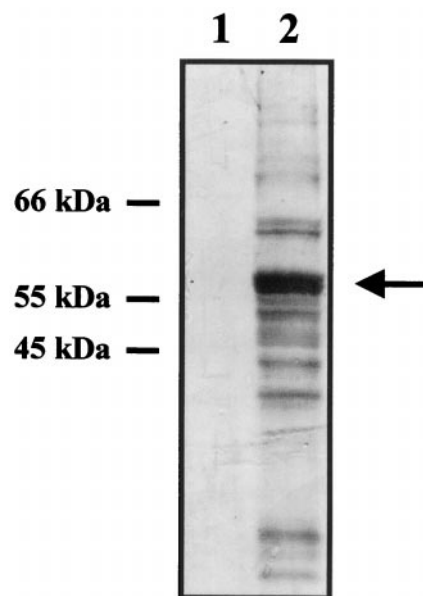


Fig. 3. Western blot analysis of ketoprofen-protein adducts. Four hours after treatment of PB-treated rat liver microsomes with 0 (1) or 2.5 mM (2) of ketoprofen glucuronide, the proteins were separated by SDS-PAGE, transferred to an Immobilon-P membrane, and probed with antiketoprofen antibodies. Amount of protein loaded was 100 μ g/lane. Arrow indicates the 56-kDa protein band.

In Situ Irreversible Binding of Ketoprofen Glucuronide to Proteins. The experiments described above showed the *in vitro* formation of adducts in subcellular fractions. To determine whether the irreversible binding of ketoprofen glucuronide also occurred in whole cells in culture, nontransfected and UGT2B1-transfected V79 cells were exposed for 24 h to culture medium containing either ketoprofen at a final concentration of 1 mM or the vehicle [5% dimethyl sulfoxide (v/v)]. We verified that the formation of ketoprofen glucuronide by the UGT2B1-transfected V79 cells was effective. Ketoprofen glucuronide (5.75 ± 0.45 nmol) was formed and secreted into the extracellular medium when the UGT2B1-transfected V79 cells were incubated for 24 h with 1 mM ketoprofen and 5 mM glucose as a precursor for UDP-

GlcUA formed intracellularly, corresponding to a concentration of about $0.5 \mu\text{M}$. We then measured the amount of 2-naphthol glucuronide released into the culture medium from control and ketoprofen-treated UGT2B1-transfected cells and the amount of irreversibly bound ketoprofen glucuronide (Table 1). Following exposure of the UGT2B1-transfected cells to ketoprofen, we observed a significant decrease of approximately 35% of 2-naphthol glucuronidation that was correlated with adduct formation.

To confirm the irreversible binding of ketoprofen glucuronide to proteins *in situ*, cells treated as described above were analyzed by immunofluorescence microscopy using anti-UGT and antiketoprofen antibodies (Fig. 6). UGT2B1-transfected V79 cells showed a typical endoplasmic reticulum fluorescence pattern, when they were stained by anti-UGT antibodies (Fig. 6C), in accordance with the membrane endoplasmic reticulum localization of UGTs. A similar distribution of the fluorescence signal was observed when the UGT2B1-transfected V79 cells were treated with ketoprofen and probed with antiketoprofen antibodies (Fig. 6A). As a control, we checked that UGT2B1-transfected V79 cells exhibited no fluorescence staining when they were not treated with ketoprofen and probed with antiketoprofen antibodies (data not shown). In addition, no fluorescence signal was observed when control V79 cells, which do not express UGTs, were exposed to 1 mM ketoprofen for 24 h and then immunolabeled with anti-UGT antibodies (data not shown) and antiketoprofen antibodies (Fig. 6B). This indicates that adduct formation occurred only when ketoprofen glucuronide was synthesized. Moreover, treatment of UGT2B1-transfected V79 cells with 1 mM ketoprofen in the presence of 2 mM 7,7,7-triphenylheptanoic acid, a strong inhibitor of UGTs (Fournel-Gigleux et al., 1989) that caused a 85% inhibition of UGT activity toward 2-naphthol, led to the abolishment of the immunofluorescent staining when antiketoprofen antibodies were used (data not shown). These results suggest that the biosynthesis of ketoprofen glucuronide is a prerequisite for ketoprofen-protein adduct

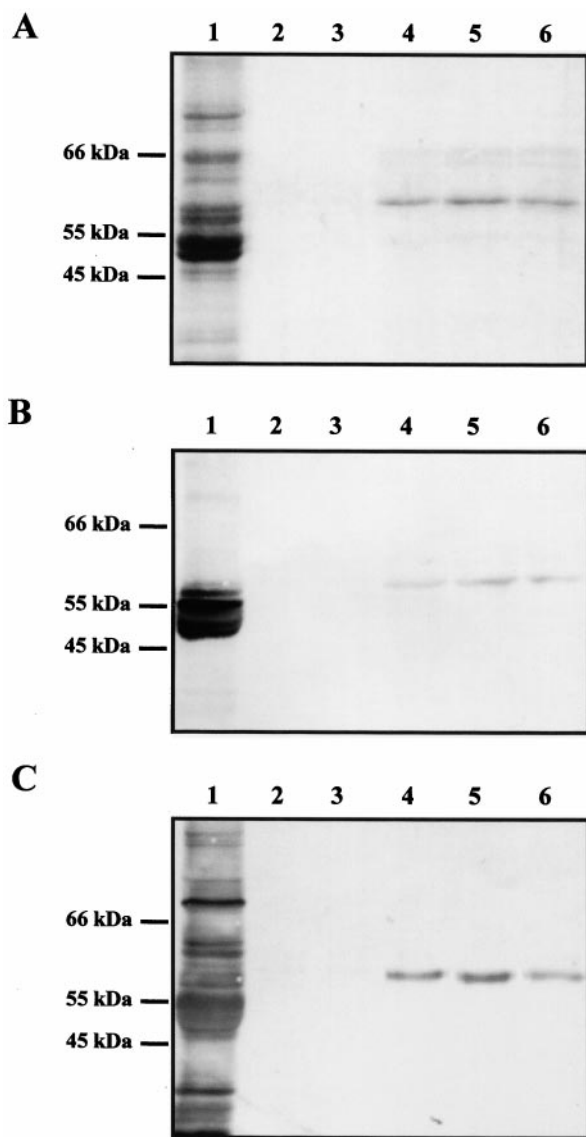


Fig. 4. Purification of UGT adducts. Four hours after treatment of PB-treated rat liver microsomes with 5 mM ketoprofen glucuronide, the microsomes containing the protein adducts were solubilized by CHAPS and applied to a DEAE-Sephacel column, as described in *Material and Methods*. Solubilized microsomal proteins (lane 1) and fractions eluted from the column with 100 mM KCl (lanes 2, 3, 4, 5, and 6) were separated by SDS-PAGE and detected by Coomassie blue (A) or transferred to an Immobilon-P membrane and probed with anti-UGT (B) and antiketoprofen (C) antibodies.

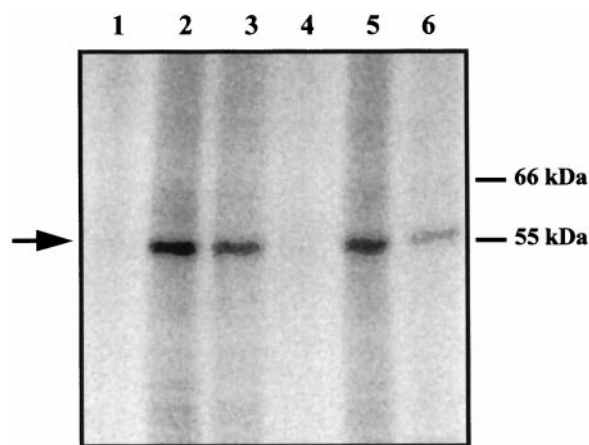


Fig. 5. Effect of ketoprofen glucuronide on the photoaffinity labeling of UGT2B7 with $[\beta\text{-}^{32}\text{P}]\text{5N}_3\text{UDP-GlcUA}$. Membrane fractions of UGT2B7-transfected HK293 cells were incubated in the absence (control and lanes 1, 2, and 3) or the presence (lanes 4, 5, and 6) of 5 mM ketoprofen glucuronide for 30 min, as described in *Materials and Methods*. Proteins ($50 \mu\text{g}$) were then photolabeled with $40 \mu\text{M}$ $[\beta\text{-}^{32}\text{P}]\text{5N}_3\text{UDP-GlcUA}$ by UV-irradiation (254 nm) for 90 s. Lanes 1 and 4 are experiments without UV-irradiation, and lanes 3 and 6 are protection experiments done in the presence of 0.4 mM unlabeled UDP-GlcUA. Labeled proteins were separated by SDS-PAGE and followed by autoradiography, as described in *Material and Methods*. Autoradiograph represents one representative experiment from two independently repeated experiments.

formation in situ and that the target proteins are mainly localized in the endoplasmic reticulum of the cells, which corresponds to the intracellular localization of UGTs.

Discussion

Because of its role in the transport of drugs and metabolites, serum albumin has been found by us and others to be a target of acylglucuronides of various NSAIDs, including ketoprofen, both in vitro and in vivo (Ojingwa et al., 1994; Presle et al., 1996). Intracellular targets of acylglucuronides have been far less investigated. Wang and Dickinson (1998) reported the presence of protein adducts (110 and 120 kDa) in rat liver homogenates with diflunisal acylglucuronide. Because these metabolites are generated by UGTs, we investigated the possibility that these enzymes may be the primary targets of acylglucuronides. Microsomal UGTs and the recombinant UGT2B1 and UGT2B7, which have been identified as the main isoforms involved in the glucuronidation of profens in rat and humans, have been considered.

Our studies revealed that ketoprofen glucuronide was a potent in vitro inhibitor of glucuronidation reaction catalyzed by human liver microsomes or by UGT2B1. We showed that this inhibition was irreversible and was related to the amount of ketoprofen glucuronide covalently bound to microsomal proteins. Relatively few amounts of ketoprofen glucuronide covalently bound to human liver microsomes or UGT2B1-V79 membrane fraction (0.25 and 0.03 nmol ketoprofen glucuronide per mg of total microsomal proteins, respectively) were able to completely abolish UGT activities in vitro. In the same way, the concentration of ketoprofen glucuronide formed endogeneously in V79 fibroblasts expressing UGT2B1 was enough to generate protein adducts and to decrease, by 35%, the glucuronidation potency of this cellular system toward 2-naphthol. These results suggested that the irreversible inhibition occurred via the covalent binding of the acylglucuronide to a key domain of the enzyme.

We provided the following experimental evidences strongly supporting this hypothesis. First, Western blot analysis using antiketoprofen antibodies revealed that several intracellular proteins bind the ketoprofen acylglucuronide, but among those was a major immunoreactive polypeptide with an apparent molecular mass (56 kDa) in the range of that of UGTs. Moreover, immunofluorescence experiments on V79 cells stably expressing UGT2B1 showed a typical endoplasmic reticulum-type distribution of the ketoprofen adducts,

which was similar to that of the UGTs. Finally, we successfully purified to apparent homogeneity UGT proteins from rat liver microsomal fractions that had reacted with ketoprofen glucuronide. The purified UGTs were recognized by both

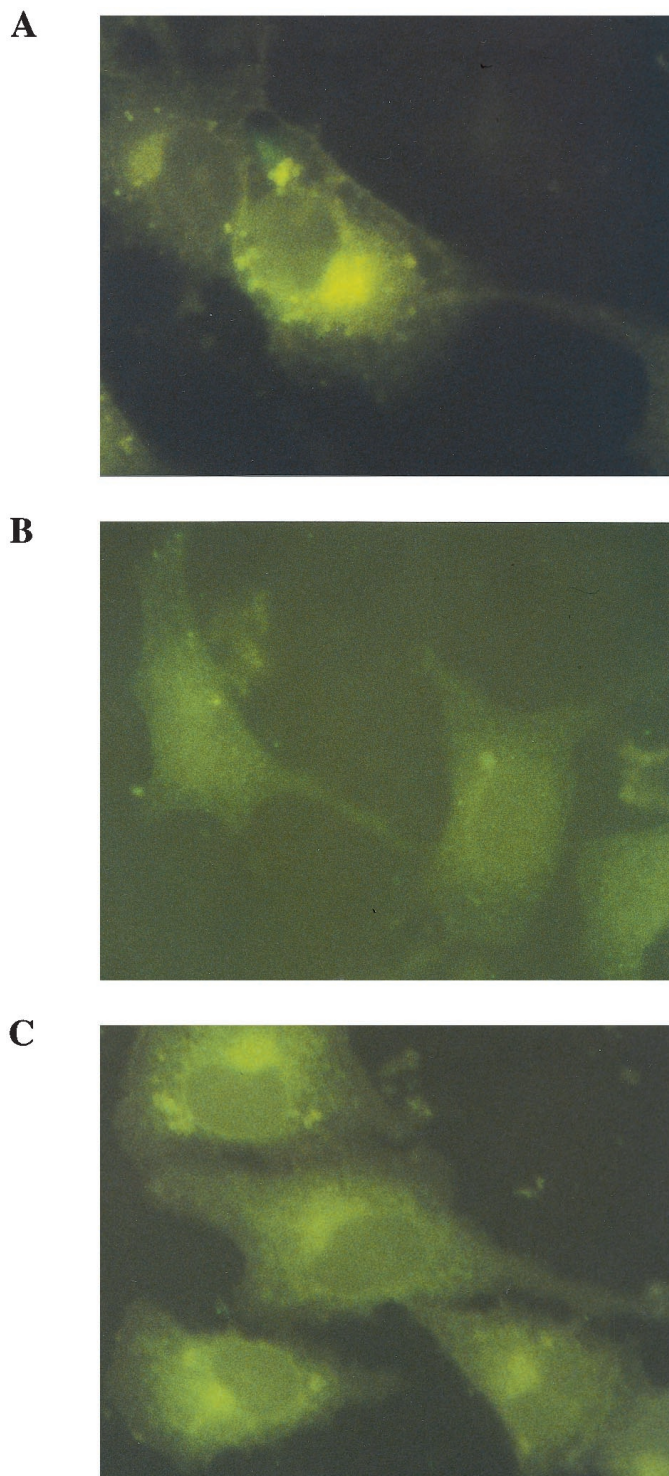


Fig. 6. Visualization by fluorescence microscopy of ketoprofen-protein adducts formed in situ. Twenty-four hours after treatment with 1 mM ketoprofen, UGT2B1-transfected (A) or nontransfected (control) (B) V79 cells were fixed, permeabilized, and stained with antiketoprofen antibodies and FITC-conjugated goat anti-rabbit IgG. UGT protein in UGT2B1-transfected cells was also visualized with anti-UGT antibodies and FITC-conjugated donkey anti-goat IgG (C). Original magnification, 1000 \times .

TABLE 1

Effect of addition of ketoprofen to culture medium on rate of glucuronidation of 2-naphthol in UGT2B1-transfected V79 cells and on formation of covalent binding of ketoprofen glucuronide to proteins

Measurement of 2-naphthyl- β -D-glucuronide formation in cultured UGT2B1-transfected V79 cells and of covalent binding of ketoprofen glucuronide to proteins were performed as described in *Materials and Methods* after treatment by dimethyl sulfoxide [0.5% (v/v)] or ketoprofen [1 mM dissolved in dimethyl sulfoxide, 0.5% (v/v)]. Results are expressed as nmol of 2-naphthol glucuronide formed/90-mm diameter dish or, in parentheses, as a percentage of residual activity (ratio of glucuronidation activity in the presence and absence of ketoprofen). Results are mean \pm S.D. of three separate experiments.

V79-UGT2B1	Glucuronidation Activity ^a	Covalent Binding ^b
No ketoprofen	43.9 \pm 5.0 (100)	0
Ketoprofen	29.3 \pm 1.9 (66.7 \pm 4.3)	30.0 \pm 4.2

^a Glucuronidation activity is expressed as nmol ketoprofen glucuronide formed/pellet \times 10 h.

^b Covalent binding of ketoprofen glucuronide to proteins is expressed as pmol ketoprofen bound/mg of protein.

antibodies raised against UGTs or ketoprofen, indicating that UGTs are targets of ketoprofen glucuronide.

The photoaffinity experiments showed that ketoprofen glucuronide competed with the radiolabeled probe, an analog of UDP-GlcUA, to bind the UGTs, providing the information that the metabolite may react with the UDP-GlcUA binding site of UGT2B7. On the other hand, ketoprofen glucuronide was able to inhibit different UGT isoforms (IC_{50} ranged from 0.5–5 mM), especially UGT1A6 (data not shown). This protein glucuronidates mainly planar and short phenols as well as naproxen, but with a low activity (Ebner and Burchell, 1993). The interaction of ketoprofen glucuronide with the active site of UDP-GlcUA, which is highly conserved among UGT isoforms (Mackenzie et al., 1997), would explain that the inhibition exerted by ketoprofen glucuronide is not specific with regard to the different UGT isoforms. Moreover, UGT2B1 and UGT2B7 are known to glucuronidate, besides NSAIDs, several types of chemically different substrates, including morphine, catechol estrogens, and androsterone. The inhibition resulting from adduct formation may therefore impair the metabolism of these physiologically active compounds.

With human serum albumin, a nucleophilic displacement of the glucuronic acid moiety leads to the covalent attachment of *R*-ketoprofen by acylation and to the release of glucuronic acid. The Schiff base mechanism of the adduct formation involves acylmigration from the carbon 1 of the sugar moiety on the other carbon atoms, subsequent opening of the sugar ring, and imine formation between the free aldehyde of glucuronic acid and the protein. Under these conditions, the acylglucuronide is bound to the protein via glucuronic acid (Presle et al., 1996). Because these two mechanisms are likely to occur simultaneously at physiological pH, we suggest that the complex irreversible inhibition, revealed by the biphasic kinetics, could reflect the existence of these reactions, which are known to proceed at different rates. Indeed, an attempt to investigate the mechanism of adduct formation using synthesized radiolabeled acylglucuronide (on the glucuronic acid moiety) was undertaken. No band could be detected upon incubation of the probe with microsomal proteins, even after 1 month of exposure. We explain this result by the fact that, either the specific activity of the radioactive metabolite was too low, or the binding proceeded via a nucleophilic displacement, which implies the release of labeled glucuronic acid. More work is needed with the use of radiolabeled ketoprofen to discriminate between a Schiff base and a nucleophilic mechanism.

Evidence of toxicity induced by electrophilic acylglucuronides has been demonstrated, especially for NSAIDs of the class of arylalkyl carboxylic acids. A high incidence of anaphylactic reactions has been described for NSAIDs, such as zomepirac, which has been removed from the market. Ketoprofen has also been reported to be responsible for such reactions, although not frequently (Tardy et al., 1989). The molecular mechanism of these immune reactions has not been elucidated yet. Because these substances are mainly metabolized into acylglucuronides in humans, the reactivity of this electrophilic species toward target proteins favored the hapten hypothesis (Spahn-Langguth and Benet, 1992). Worrall and Dickinson (1995) showed that diflunisal acylglucuronide was an immunogen in rats, supporting the hypothesis that covalent modification of macromolecules by acylglu-

curonides can lead to the production in vivo of circulating antibodies, which may be involved in immune responses and drug hypersensitivity. Indeed the presence of UGTs as hepatocellular autoantigens has been described in patients with autoimmune or viral hepatitis (Manns and Obermayer-Straub, 1997). The question that arises from these data concerns the consequences of the formation of UGT adducts and the possibility of modified UGTs acting as autoantigens in vivo situations in humans. Work is in progress to find out whether such a mechanism accounts for the immune reactions observed in some patients taking carboxylic NSAIDs.

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