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N-Glucuronidation of the antiepileptic drug retigabine: results from studies with human volunteers, heterologously expressed human UGTs, human liver, kidney, and liver microsomal membranes of Crigler-Najjar type II

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Abstract

Retigabine (D-23129), an *N*-2-amino-4-(4-fluorobenzylamino)phenylcarbamine acid ethyl ester, is a novel antiepileptic drug which is currently in phase II clinical development. This drug undergoes N-glucuronidation. We aimed to identify the principal enzymes involved in the N-glucuronidation pathway of retigabine and compared our findings with those obtained from human liver (a pool of 30 donors) and kidney microsomes (a pool of 3 donors) and with results from a human absorption, distribution, metabolism, and excretion study upon administration of 200 μCi of [^{14}C]-D-23129. Essentially, microsomal assays with UGT1A1 produced only one of the 2 *N*-glucuronides, whereas UGT1A9 is capable of forming both *N*-glucuronides. The rates of metabolism for UGT1A9, human liver microsomes, and UGT1A1 were 200, 100, and 100 pmol *N*-glucuronide per minute per milligram of protein, respectively. At the 50 μmol/L uridine diphosphate glucoronic acid (UDPGA) concentration, UGT1A4 also catalyzed the N-glucuronidation of retigabine, the rates being approximately 5 and 6 pmol/(min · mg protein). With UGT1A9, the production of metabolites 1 and 2 proceeded at a K_m of 38 ± 25 and 45 ± 15 μmol/L, whereas the K_m for retigabine N-glucuronidation by human liver microsomal fractions was 145 ± 39 μmol/L. Furthermore, a V_{max} of 1.2 ± 0.3 (nmol/[min · mg protein]) was estimated for human liver microsomes (4 individual donors).

We investigated the potential for drug-drug interaction using the antiepileptic drugs valproic acid, lamotrigine, the tricyclic antidepressant imipramine, and the anesthetic propofol. These are commonly used medications and are extensively glucuronidated. No potential for drug-drug interactions was found at clinically relevant concentrations (when assayed with human liver microsomes or UGT1A9 enzyme preparations).

Notably, the biosynthesis of retigabine–*N*-glucuronides was not inhibited in human liver microsomal assays in the presence of 330 μmol/L bilirubin, and glucuronidation of retigabine was also observed with microsomal preparations from human kidney and Crigler-Najjar type II liver. This suggests that lack of a particular UDP-glucuronosyltransferase (UGT) isoform (eg, UGT1A1 in kidney) or functional loss of an entire UGT1A gene does not completely abolish disposal of the drug. Finally, chromatographic separations of extracts from microsomal assays and human urine of volunteers receiving a single dose of ^{14}C-retigabine provided clear evidence for the presence of the 2 *N*-glucuronides known to be produced by UGT1A9. We therefore suggest N-glucuronidation of retigabine to be of importance in the metabolic clearance of this drug.

1. Introduction

Retigabine (D-23129), an *N*-2-amino-4-(4-fluorobenzylamino)phenylcarbamine acid ethyl ester (see Fig. 1), is a new antiepileptic drug which is in clinical phase II development. It exerts potent anticonvulsant effects in a wide variety of animal models of epileptic seizures including models with electrical and chemical induction of convulsion and in genetic models of epilepsy [1-4]. Unlike standard anticonvulsants, retigabine is more active in the amygdala kindling model, which is believed to be a predictive model of complex partial seizures in humans, when compared with models of generalized seizures like the maximal electroshock seizure threshold test. Importantly, the drug exerts antiepileptogenic

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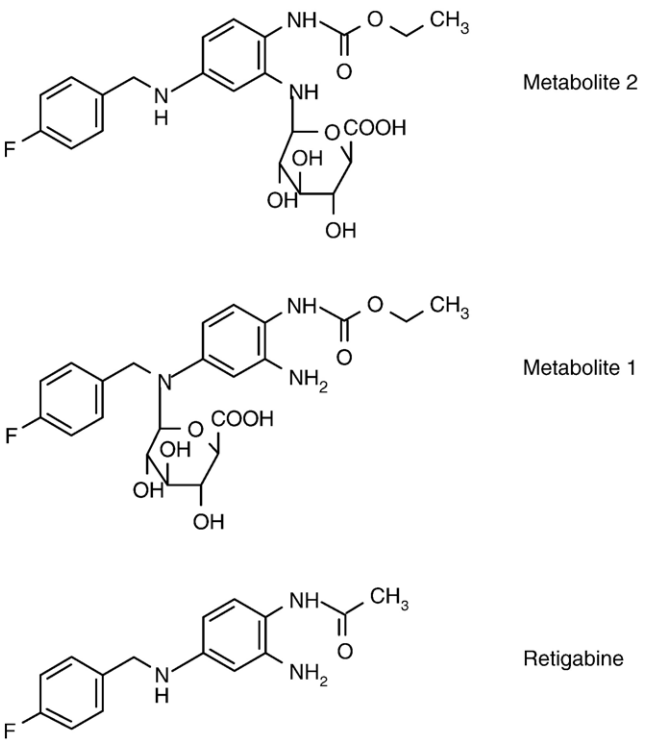

Fig. 1. The structure of retigabine and its 2 major *N*-glucuronides.

effects in low doses and no dependence liability could be observed during chronic treatment.

It was shown that retigabine interacts with specific potassium M channels and particularly the KCNQ2/3 potassium channel heteromultimere, which are involved in the control of the excitability of neuronal cells [5,6]. Thus, retigabine can be classified as M-channel agonist, and its mode of action targets novel and powerful cellular endpoints for the treatment of epilepsy. This particular mode of action may be termed as *selective neuronal potassium channel opener*, as suggested by Kornhuber et al [7]. Furthermore, retigabine potentiated γ-aminobutyric acid–induced currents in rat cortical neurons in a concentration-dependent manner. These effects were seen at concentrations of 10 μmol/L, whereas the potassium channel opening effects can be seen at concentrations as low as 0.1 μmol/L [1,8].

The metabolism of retigabine is dominated by N-glucuronidation [9]. The main biotransformation pathway in rat involves N-dealkylation and N-glucuronidation of the unchanged parent compound, with *N*-glucuronides being the major metabolites in plasma and bile [9]. In rats, the pharmacokinetics of retigabine was linear at pharmacodynamically effective doses [10].

In dogs, the metabolism of retigabine is less complex with high levels of systemically available *N*-glucuronides. In contrast to rats, neither acetylation nor N-dealkylation products could be identified in plasma or urine samples [9].

In vitro investigations with fresh human liver slices and human liver microsomes are suggestive for the production of *N*-glucuronides with little or no evidence for cytochrome P450 monooxygenase–facilitated metabolism of retigabine [9].

In view of its extensive glucuronidation, we wished to investigate the contribution of individual UDP-glucuronosyltransferase (UGT) isozymes using cloned human UGTs expressed in V79 cell lines.

We further compared findings from individual enzyme preparations with those obtained from human liver and kidney microsomes (which lack UGT1A1) and with results from a human absorption, distribution, metabolism, and excretion study upon administration of 200 mg of [^{14}C]-labeled retigabine.

We finally investigated the biotransformation of retigabine with liver tissue from a single pediatric patient diagnosed with Crigler-Najjar type II syndrome. This patient had complete functional loss of the entire UGT1A gene and received liver transplantation.

Assuming competitive enzyme kinetics, we addressed the issue of drug-drug interactions of commonly prescribed and/or coadministered antiepileptic drugs including valproic acid and lamotrigine, as well as the tricyclic antidepressant imipramine and the anesthetic propofol. Particularly, these drugs are also extensively glucuronidated.

2. Material and methods

2.1. Chemicals

Retigabine (D-23129) was synthesized by ASTA Medica (Dresden, Germany) and was of more than 99% purity (batch no. 9805102, Certificate of Analysis FAS/980137).

^{14}C-Retigabine with a specific activity of 333 MBq/mmol (= 9 mCi/mmol) was synthesized by Amersham (Little Chalfont, Buckinghamshire, England) and was of 98.5% purity as determined by thin layer chromatography using a cyclohexane/acetone/0.88 ammonia (7:6:1) solvent system.

Unless otherwise stated, all other reagents were of highest purity and were purchased from Sigma Aldrich (Seelze, Germany) or Becton Dickinson (Heidelberg, Germany).

2.2. Microsomal preparations of human liver and kidney microsomes

Approval for the use of human kidney (a pool of 3 donors) and liver tissue (a pool of 30 donors) was obtained from the ethical committee of Ninewells Hospital and Medical School of the University of Dundee (Brian Burchell) and the Medical School of Hannover (Jürgen Borlak), respectively.

Microsomal preparations were obtained by ultracentrifugation as reported previously [11]. Notably, human kidney microsomes were used, because kidney cells do not express UGT1A1. Microsomal assays were done with incubation mixture consisting of 300 μmol/L ^{14}C-radiolabeled retigabine, 7 mmol/L $MgCl_2$, 2.5 mmol/L uridine diphosphate glucoronic acid (UDPGA) in a 0.1 mmol/L Tris-HCl buffer

adjusted to a pH of 8.0. The reaction was initiated by addition of 0.2 mg microsomal protein per milliliter, and incubations were performed for 40 minutes at 37°C in a shaking water bath. The final volume of the reaction mixture was 0.1 mL, and the reaction was stopped with an equivalent amount of acetonitrile. To allow for protein precipitation, the samples were kept in a refrigerator at −20°C for 20 minutes. Then, sample analysis was done by radiochemical high-performance liquid chromatography (HPLC) as detailed below (see Analytical HPLC). The Crigler-Najjar type II liver tissue stems from a male patient and was diagnosed with complete deletion of bases 510 to 512, which disrupts a diphenylalanine repeat. This genetic alteration resulted in a complete functional loss of the entire UGT1A gene.

2.3. Tissue culture

The cloning and expression of human UGTs in V79 cell lines are reported in detail in Refs. [12-14]. The following UGTs were used: UGT1A1, UGT1A4, UGT1A6, UGT1A9, UGT2B7, UGT2B15. Recombinant Hek293 cells expressing UGT2B15 were a kind gift from Dr T Tephly (University of Iowa), and V79 cell lines heterologously expressing human UGTs were maintained under optimized constant selection concentrations of geneticin (G418, Invitrogen, Karlsruhe, Germany) as reported in detail by Ethell et al [15]. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco Life Technologies). Cells were harvested after washing twice with ice-cold phosphate buffered saline by gently scraping the monolayer into a volume of 2.5 mL of ice-cold phosphate buffered saline. Cells in suspension were pelleted by centrifugation at 1000 × *g* for 3 minutes and sonicated before use. Cells were stored at −80°C until used in assays.

2.4. Retigabine glucuronidation assay

Retigabine stock solution was prepared as described previously [16] and was used at a concentration of up to 1000 μmol/L. The liver and kidney microsomal protein was adjusted to 0.2 mg of protein per milliliter of buffer, and the incubation buffer consisted of 0.1 mL Tris-HCL pH 8.0. The cofactor solution contained 10 mmol/L ascorbic acid, 7 mmol/L $MgCl_2$, and 2 mmol/L UDPGA. The volume of the incubation mixture was 1 mL, and assays were done at 37°C for 40 minutes, and the reactions were stopped with an equivalent amount of acetonitrile. Samples were transferred to a refrigerator at 4°C, and protein precipitation was allowed over a period of 30 minutes. Samples were centrifuged at 10 000 × *g* at 4°C for 30 minutes, and supernatants were removed and reduced in volume for further HPLC analysis as detailed below.

2.5. Drug-drug interaction/competition assays

Assay conditions were identical as detailed above with ascending concentrations of the competitor substrate, and this included concentrations at clinically relevant plasma levels. We used valproic acid (0.5-5 mmol/L), lamotrigine (1-500 μmol/L), imipramine (1-1000 μmol/L), propofol (5.0-750 μmol/L), and bilirubin (1-500 μmol/L) as an endogenous substrate.

2.6. Assay with expressed UGT isoforms

This was done with the optimized conditions described by Ethell et al [15]. The following substrates were used to assay for heterologously expressed UGT isoforms: octylgallate for UGT1A1, entacpone for UGT1A4, 1-naphthol for UGT1A6, propofol for UGT1A9, hyodeoxycholic acid for UGT2B7, 8-hydroxyquinoline for UGT2B15, and 1-naphthol for human liver microsomes.

2.7. Analytical HPLC

Test article, urine, and plasma samples were analyzed by radiometric HPLC using the following conditions and parameters:

KONTRON HPLC system 400 with data system 450-MT2 (KONTRON Instruments, Neufahrn, Germany) consisting of 2 HPLC pumps 420, gradient mixer M 800, autosampler 465 (adapted to inject from 50 to 1250 μL) and UV detector 432, adjusted at 220 nm, range 0.5, and response time 2.0 seconds.
Radiomonitor LB 507 B (Berthold, Bad Wildbad, Germany), adjusted at a response time of 1 second and peak half-width of 30 seconds, using a solid scintillator flow cell YG-150 U4 at a range of up to 10^4 dpm. Background, less than 500 cpm. Column: Kromasil C18, 5 μm (250 × 4.6), with guard (30 × 4.6) (Phenomenx, Aschaffenburg, Germany); volume of injection, 100 to 800 μL; flow rate, 0.5 mL/min.

In both HPLC systems (analytical and semipreparative), the same mobile phases and gradient were used.

Mobile phase A: 20 mmol/L KH_2PO_4 solution, adjusted with NaOH solution (1 mol/L) to pH 7.2; mobile phase B: acetonitrile/water 90/10 (v/v).
Gradient:

Time (min)	Eluent A (%)	Eluent B (%)	Time (min)	Eluent A (%)	Eluent B (%)
0	100	0	210	11	89
20	100	0	211	100	0
70	90	10	231	100	0
196	11	89			

The chromatography was performed at room temperature (21°C-23°C).

2.8. Electrospray tandem mass spectroscopy

The samples were analyzed using the HPLC system linked on-line to a Quattro tandem quadrupole mass spectrometer equipped with a Megaflow electrospray interface (Micromass, Manchester, UK). For LC-MS analysis, the analytical HPLC system was applied using modified mobile phases.

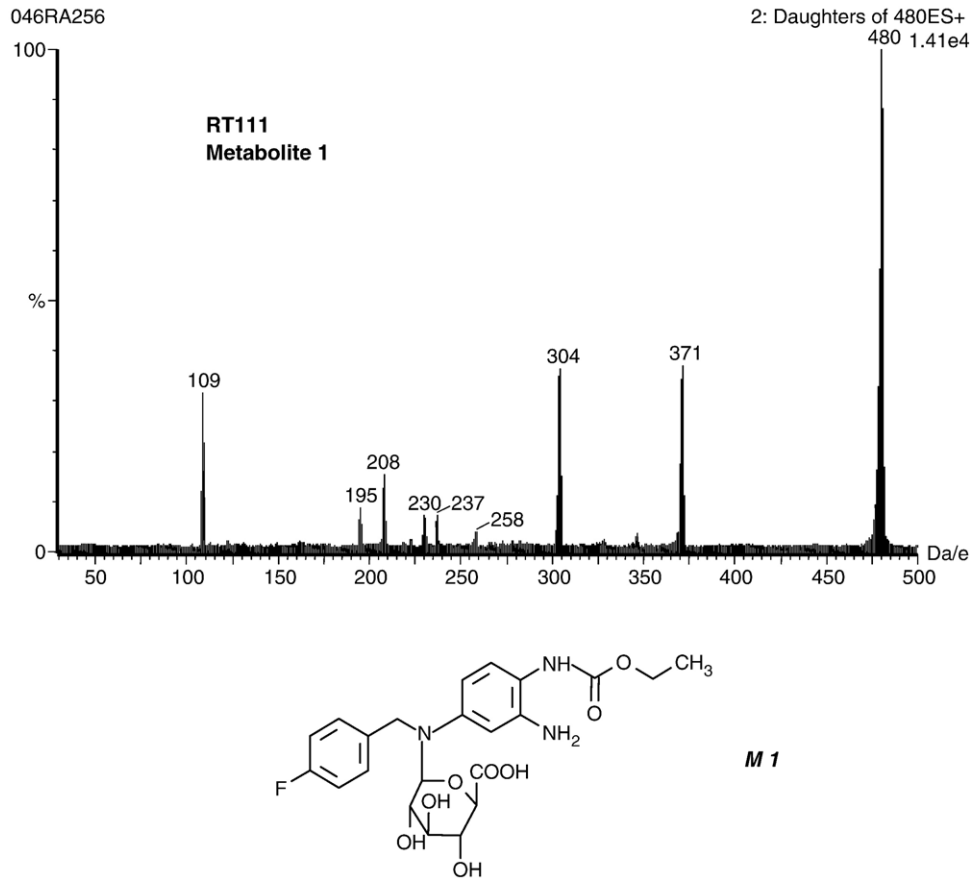

Fig. 2. Production spectrum of the protonated molecular ion $[M + H]^+$ at m/z 480 of M1 with RT111 obtained by on-line HPLC/ESI-MS/MS of urine from subject 101M, collected 2 to 4 hours after administration.

Mobile phase A: water containing 15 mmol/L ammonium acetate adjusted with NH_3 solution (1:10, v/v) to pH 7.4. Mobile phase B: 90% acetonitrile/10% water (v/v) containing 1 mmol/L ammonium acetate.

After UV and radiometric detection, the HPLC effluent was split postcolumn to 60 μL/min and introduced into the electrospray source (source temperature, 80°C). Mass spectra were acquired as profile data with 16 points per dalton, alternately in positive and negative ion mode. The nebulizer and bath gases were nitrogen delivered at flow rates of 12 and 250 L/h, respectively. Electrospray mass spectra were acquired by scanning from m/z 125 to m/z 900 in 3 seconds in MS1 mode. Product ions formed by collision-induced dissociation from protonated molecular ions were generated in positive and negative ion mode with argon as collision gas at 2.1×10^5 mbar and a collision energy of 50 eV (scan rate, 250 U/s) at a cone voltage of 30 or 50 V.

2.9. ^{14}C HPLC assay for the determination of UDP-glucuronosyltransferase activity

Two different assays were used:

1. A universal radio chromatographic method for the determination of UDP-glucuronosyltransferase activity according to Ethell et al [15]. The assay was done with the equipment and instrumental settings reported previously [15] with ^{14}C-UDPGA purchased from NEN DuPont (Stevenage, Hertfordshire, UK).
2. An HPLC assay for the determination of retigabine glucuronides according to Hiller et al [16] with modifications as described below. The assay was done with the equipment and instrumental settings as reported previously [16] with ^{14}C-retigabine at a specific activity of 333 MBq/mmol (= 9 mCi/mmol) purchased from Amersham.

The analytical system for the determination of retigabine and its glucuronides consisted of an HP1100 HPLC system (Hewlett Packard, Waldbronn, Germany) connected to a Kromasil C18, 5-μm (250 × 4.6 mm) column and a guard column with a pore size of 5 μm and a dimension of 30 × 4.6 mm (Phenomenex, Höfbach, Germany). The mobile phase consisted of eluent A (20 mmol/L KH_2PO_4, pH 7.2) and eluent B (90% acetonitrile/10% water [v/v]) at a flow rate of 0.5 mL/min and a column temperature of 20°C to 22°C. Linear gradient was programmed as follows: 0, 50, 75, 78, 86, and 96 minutes with 10%, 42%, 73%, 89%, 10%, and 10% of eluent B, respectively. The sample temperature was 4°C and the injection column was

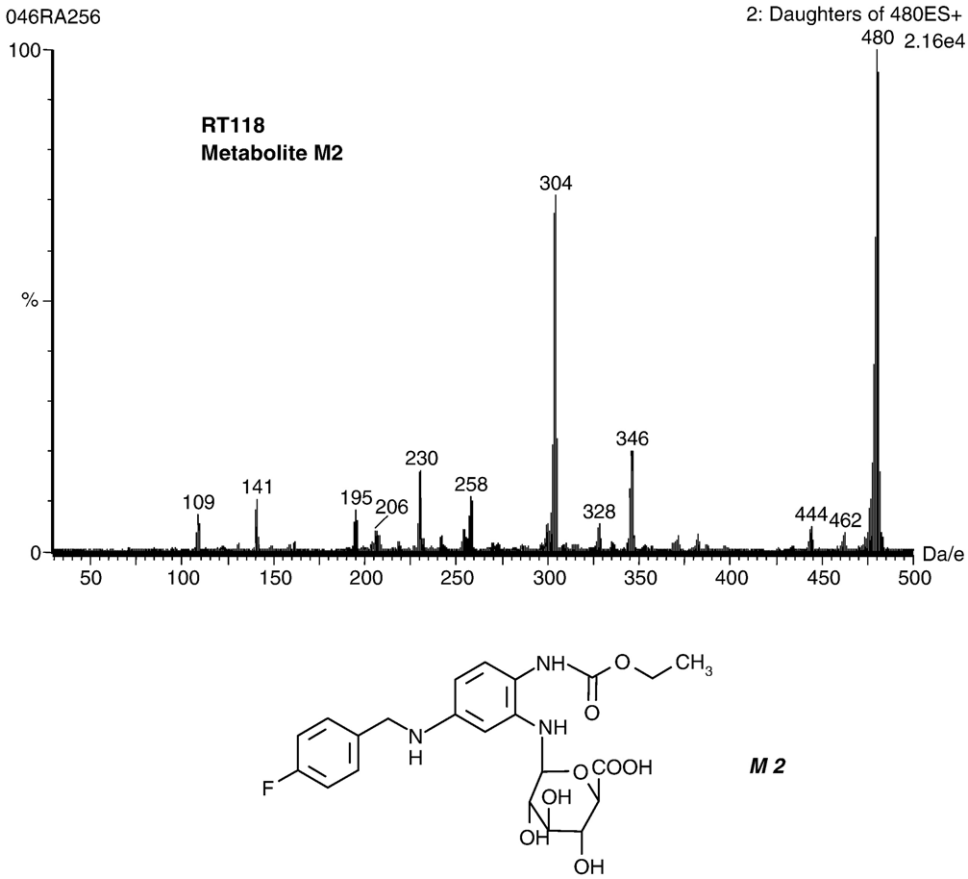

Fig. 3. Production spectrum of the protonated molecular ion $[M + H]^+$ at m/z 480 of M2 with RT118 obtained by on-line HPLC/ESI-MS/MS of urine from subject 101M, collected 2 to 4 hours after administration.

200 μL. Detection of analyte was with a diode array detector (wave length, 220 nm; slit, 8 nm; bandwidth, 40 nm).

Glucuronide concentrations were derived on the basis of external calibration using retigabine as the reference compound. Reaction velocities of product formation were calculated as picomoles of product formed per minute per milligram of microsomal protein (pmol/min · mg).

3. Results

3.1. Studies with human volunteers

The metabolism of retigabine in humans was investigated after a single oral administration of a 200-mg dose of [^{14}C]-retigabine to 6 healthy male subjects (study 3065A1-108-US). Plasma, feces, and urine samples were collected before and up to 240 hours after dose administration and were analyzed by HPLC/tandem mass spectroscopy (HPLC/MS/MS) as detailed in Material and Methods. Total radioactivity was measured by liquid scintillation counting.

The mean recovery of radioactivity was 97.9% at 240 hours after oral dose administration. Within 72 hours after administration, 84% of the orally administered dose was recovered in the urine, and 9% was recovered in the feces over 96 hours. The high recovery in urine suggests extensive absorption of the drug. In the following days, an additional 5% of the dose was found in the feces. Retigabine was mainly excreted by the kidneys as unchanged compound and as 7 metabolites (see Fig. 6, lower panel); the major ones being *N*-glucuronides termed metabolite 1 (M1) (see also Figs. 1 and 2; note M1 has a retention time [RT] of 111 minutes [RT111]) and glucuronide metabolite 2 (M2) (see also Figs. 1 and 3; note M3 has an RT of 118 minutes [RT118]). Furthermore, an *N*-acetyl derivative of retigabine was present in human plasma and urine as well (see next page for a discussion on the relative importance of this additional phase II metabolite).

In the feces, unchanged compound and 3 metabolites were observed. None of the metabolites in feces were found in plasma or urine. These metabolites may come from degradation of retigabine by intestinal enzymes. In plasma, a total of 6 radioactive peaks were detected with the *N*-glucuronides being dominant. Total plasma radioactivity peak levels were about 12-fold higher than retigabine C_{max}, predominantly explained by the *N*-glucuronides, and the radioactivity was eliminated at the same rate as the *N*-acetyl derivative of retigabine.

The mean blood/plasma ratios of ^{14}C-retigabine–derived radioactivity ranged between 0.55 and 0.68, indicating no preferential partitioning of radioactivity into whole-blood constituents.

Table 1
Summary of major biotransformation products of retigabine in human volunteers

Component	Plasma[a]	Urine[b]
Parent	2805	36%
M1	[c]	2%
M2	[c]	16%
AWD21-360	4009	18%

[a] Mean geometric AUC in ng · h/mL.
[b] Results represent the percentage of administered dose after a single oral administration of a 200-mg dose of [^{14}C]retigabine.
[c] Total plasma radioactivity of *N*-glucuronides was about 12-fold higher than retigabine C_{max}.

Furthermore, AWD21-360, the *N*-acetyl metabolite of retigabine, was rapidly formed and cleared at the same rate as retigabine. At steady state, the mean AWD21-360 maximum concentrations (C_{max}) and total exposure data (in terms of area under the curve [AUC] values) ranged between about 70% and 90% (for C_{max}) and between 120% and 160% (for AUC values) of the corresponding mean values for retigabine.

The urinary excretion of AWD21-360 in healthy male subjects (over 72 hours) as measured by a single oral administration of a 200-mg dose of [^{14}C]-retigabine amounted to 18% of the administered dose. Similar to the parent drug retigabine, 2 distinct *N*-glucuronides of the *N*-acetyl metabolite could also be detected in human urine which aggregate to 4% (*N*2-glucuronide) and 2% (*N*4-glucuronide) of the administered dose, respectively.

Therefore, in human volunteers, retigabine was found to be metabolized mainly by N-glucuronidation, and there was no evidence for cytochrome P450 monooxygenase–catalyzed reactions (see also Table 1 for a summary of the major biotransformation products of retigabine in human volunteers). We now report the identification of glucuronides by mass spectrometry followed by studies which show that the glucuronidation is catalyzed by several uridine diphosphate glucuronyl transferase isozymes, as detailed below.

3.2. Electrospray MS/MS of metabolites 1 and 2

Product ion spectrum of the protonated molecular ion $[M + H]^+$ at m/z = 480 of metabolites 1 (RT111) and 2 (RT118) is given in Figs. 2 and 3. As depicted in Fig. 2, the product ion spectrum is a superposition of the fragmentation of the protonated glucuronide and the aglycon, A. The fragmentation of the protonated glucuronide $[M + H]^+$ at m/z = 480 of metabolite 1 (RT111) can be explained as follows.

Loss of the glucuronide moiety, that is, $[M + H - 176]^+$ leads to m/z = 304.

Cleavage of the benzylic bond (C–N bond) can occur either with charge retention leading to $[M + H - FC_6H_4CH_2]^+$ = m/z 371 or with charge migration leading to $[FC_6H_4CH_2]^+$ = m/z 109. After loss of the glucuronide moiety, the resulting protonated aglycon, $[A + H]^+$, at m/z = 304, may undergo further fragmentation leading to the following fragments: m/z = 258: $[A + H - C_2H_5OH]^+$ (loss of ethyl alcohol); m/z = 230: $[A + H - C_2H_5OOCH]^+$ (loss of ethylformiate); m/z = 195: $[A + H - FC_6H_4CH_2]^+$ (loss of fluorobenzyl radical); m/z = 109: $[FC_6H_4CH_2]^+$ (formation of a fluorobenzyl cation).

Likewise, the product ion spectrum of the protonated molecular ion $[M + H]^+$ at m/z = 480 of metabolite 2 (RT118; see Fig. 3) can be explained as a superposition of the fragmentation of the protonated glucuronide and the aglycon, A, as follows.

Loss of the glucuronide moiety, that is, $[M + H - 176]^+$ leads to m/z = 304, m/z = 462: $[M + H - H_2O]^+$; m/z = 444: $[M + H - 2\ H_2O]^+$; m/z = 346, and cleavage of the glucuronic acid, for example, m/z = 328 $[346 - H_2O]^+$.

After loss of the glucuronide moiety, the resulting protonated aglycon, $[A + H]^+$, at m/z = 304, will undergo the same fragmentation as described for metabolite 1, for example, m/z = 258: $[A + H - C_2H_5OH]^+$ (loss of ethyl alcohol); m/z = 230: $[A + H - C_2H_5OCHO]^+$ (loss of ethylformiate); m/z = 195: $[A + H - FC_6H_4CH_2]^+$ (loss of ethylformiate); m/z = 109: $[FC_6H_4CH_2]^+$; m/z = 141 cannot be explained.

Table 2
Summary of retigabine UGT screening assays

UGT Source	50 μmol/L UDPGA		2 mmol/L UDPGA		Controls: activity with marker substrates[a]	
	M1	M2	M1	M2		
UGT1A1	ND	0.024	ND	0.098	Octylgallate	0.64
UGT1A4	0.0005	0.0006	ND	ND	Entacapone	0.349
UGT1A6	ND	ND	ND	ND	1-Naphthol	1.32
UGT1A9	0.024	0.034	0.145	0.182	Propofol	1.21
UGT2B7	ND	ND	ND	ND	Hyodeoxycholic acid	0.164
UGT2B15	ND	ND	ND	ND	8-Hydroxyquinoline	0.144
Human liver microsomes[b]	0.007	0.010	0.100	0.117	1-Naphthol	2.0 ± 0.2
Human liver microsomes[b]	–		–		Bilirubin (UGT1A1)	0.5 ± 0.1
Human liver microsomes[b]	–		–		Imipramine (UGT1A4)	0.4 ± 0.1
Human liver microsomes[b]	–		–		Propofol (UGT1A9)	0.2 ± 0.1

All activities are expressed as nanomoles per minute per milligram of protein (n = 2 independent experiments, mean values). ND indicates not detected.
[a] At 2 mmol/L UDPGA.
[b] n = 4 individual donors.

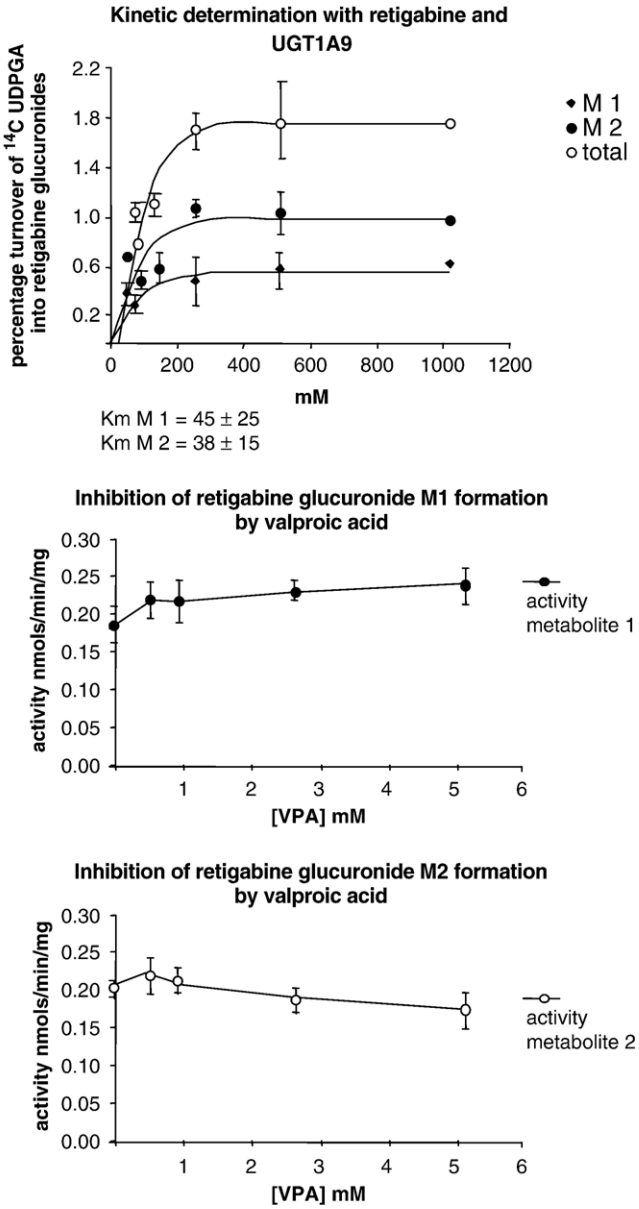

Fig. 4. Enzyme kinetics with retigabine, valproic acid, and UGT1A9.

Notably, metabolites 1 and 2 are isomers differing in the position of the glucuronidation site. Both isomers and the position of the glucuronidation site can be distinguished by mass spectrometry, which is rarely the case.

3.3. Assays with human liver and kidney microsomes and heterologously expressed UGT isoforms

Incubation experiments were done with cloned human UGTs expressed in V79 and HEK293 cell lines (UGT1A1, UGT1A4, UGT1A6, UGT1A9, UGT2B7, UGT2B15) and human liver microsomes (a pool from 30 donors) as well as human kidney microsomes (a pool of 3 donors). The incubation assay at 50 μmol/L and 2 mmol/L radioactive UDPGA shows that retigabine is metabolized by the isoforms UGT1A1, UGT1A4, UGT1A9, and by human liver and kidney microsomes. The rates of activities of the various isozymes are expressed as nanomoles of product formation per minute of incubation per milligram of total protein and are given in Table 2 as the mean of 2 independent incubation assays. Therefore, this screening enabled identification of major UGT isoforms involved in the glucuronidation of retigabine, although we were unable to normalize the retigabine N-glucuronidation activity to the amount of expressed UGT isoform. As a consequence, a direct comparison of UGT isoforms based on rates of activity is not really possible.

Nonetheless, there is evidence for UGT1A1 to produce only one of the 2 *N*-glucuronides, whereas UGT1A4 and UGT1A9 are capable of forming both *N*-glucuronides of retigabine.

3.4. In vitro interaction studies with valproic acid, lamotrigine, propofol, and imipramine

Kinetic studies were done with UGT1A9, that is, a major isoform involved in the N-glucuronidation of retigabine (n = 8 individual experiments). The results are presented in Fig. 4, and we determined the K_m's for both glucuronides which were in the order of 45 ± 25 and 38 ± 15 μmol/L for retigabine glucuronide M1 and M2, respectively. These estimates are based on the production of radiolabeled glucuronides of retigabine with assay conditions described in Material and Methods (see also Ref. [15]). Notably, the K_m for retigabine N-glucuronidation by human liver microsomal fractions was 145 ± 39 μmol/L and we estimated a V_{max} of 1.2 ± 0.3 (nmol/[min · mg protein]). Valproic acid had basically no effect on the glucuronidation of retigabine (K_m = 2 mmol/L for valproic acid and 45 μmol/L for retigabine). As shown in Fig. 4 (lower part), retigabine glucuronidation proceeded unchanged at a substrate concentration of up to 5 mmol/L valproic acid.

In addition, we investigated the potential for drug-drug interaction with propofol. This widely used anesthetic is subject to considerable glucuronidation as well.

The results of inhibition of UGT1A9-catalyzed propofol glucuronidation by retigabine are presented in Fig. 5,

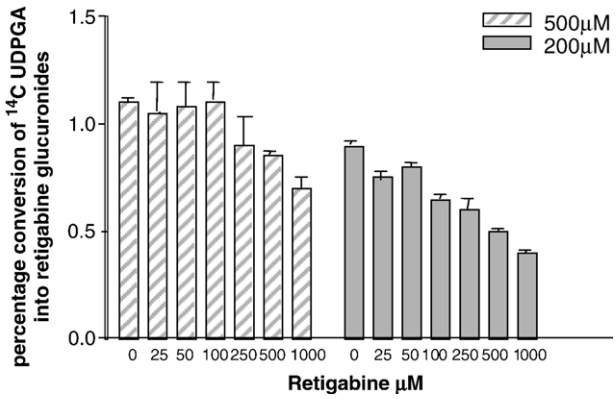

Fig. 5. Inhibition of UGT1A9 propofol glucuronidation by retigabine.

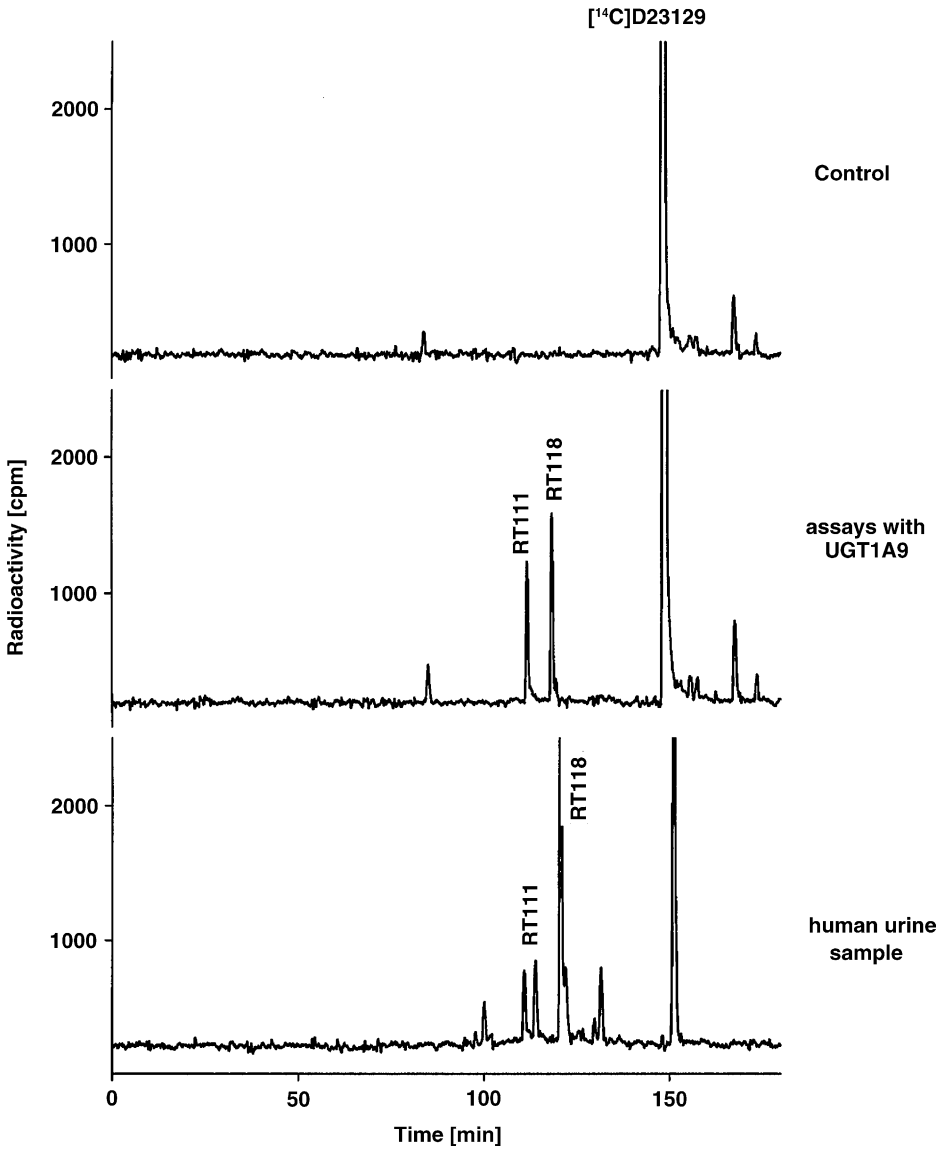

Fig. 6. Comparison of metabolite profiles of an assay with UGT1A9 and a urine sample from subject 105M, collected 0 to 2 hours after a single oral administration of 200 mg [^{14}C]retigabine. Note that M1 (RT111) and M2 (RT118) produced by UGT1A9 were found in urine samples of human volunteers receiving a single dose of 200 mg ^{14}C-labeled retigabine.

suggesting the potential of interaction of propofol with retigabine, probably only at very high retigabine concentrations of 500 and 1000 μmol/L. Again, inhibition of propofol glucuronidation by retigabine could only be achieved at approximately 400-fold higher concentrations, as compared to clinical relevant plasma values.

Because of the frequently required coadministration of antiepileptic drugs and the relatively similar metabolic pathways (ie, predominantly glucuronidation), we investigated the potential for a drug-drug interaction with lamotrigine as a further substrate but did not find the production of the *N*-glucuronides of retigabine to be inhibited at a lamotrigine substrate concentration ranging from 1 to 500 μmol/L (data not shown).

Finally, the tricyclic antidepressant imipramine was used in our in vitro competition assays to estimate the potential of drug-drug interaction with retigabine. An inhibition in the glucuronidation of retigabine was observed with a K_i of 2.4 and 1.7 mmol/L for both glucuronides, respectively. Usually, therapeutic plasma concentrations of imipramine are at least 1000-fold lower than these K_i's [17]. In humans, UGT1A1 and UGT1A4 are involved in bilirubin glucuronidation. Hence, we tested the inhibition of retigabine N-glucuronidation by bilirubin. The K_m for bilirubin glucuronidation for recombinant UGT1A1 was reported to be 2.5 μmol/L and thus 60-fold smaller than the K_m for retigabine N-glucuronidation with human liver microsomes, as detailed above. We expected N-glucuronidation of retigabine to be decreased and found inhibition by 25% $\pm$ 7% of control activity in the presence of 330 μmol/L bilirubin.

We also investigated the glucuronidation of retigabine in microsomal assays with human kidney (a pool of 3 donors)

and Crigler-Najjar type II liver tissue from a single male patient. Crigler-Najjar type II liver microsomes successfully produced retigabine glucuronides. With human kidney microsomes, metabolism of retigabine proceeded with the production of both glucuronides, whereas in microsomal assays with the Crigler-Najjar type II liver, production of only one of the *N*-glucuronides was observed. This suggests that lack of certain UGT isoforms or functional loss of the entire UGT1A gene does not completely impair retigabine glucuronide formation.

3.5. In vitro–in vivo comparisons

In clinical trials, no interaction with food was shown and retigabine does not alter the pharmacokinetics of ethinyl estradiol/norgestrel oral contraceptives [18,19]. Furthermore, no pharmacokinetic interaction with phenobarbital was observed, suggesting that no dosage adjustment is necessary when retigabine and phenobarbital are coadministered [20]. Although the pharmacokinetics of retigabine is not altered by lamotrigine, the lamotrigine clearance appeared to be slightly increased and terminal half-lives appeared to be slightly decreased under steady-state concentrations of retigabine [21].

The retigabine pharmacokinetics was unaltered by valproic acid, topiramate, phenytoin, or carbamazepine, and retigabine does not alter the pharmacokinetics of these drugs in patients with epilepsy. Phenytoin and carbamazepine, however, moderately increase the clearance of retigabine. Thus, increased retigabine doses may be required when coadministered with phenytoin and/or carbamazepine, and caution should be taken when reducing or removing coadministered carbamazepine and phenytoin as retigabine concentrations may increase [22].

3.6. Comparison of in vitro data with extracts of human urine upon administration of ^{14}C-retigabine

We also compared the metabolite pattern from microsomal assays with UGT1A9 with those obtained from a human absorption, distribution, metabolism, and excretion study. The HPLC traces of extracts from microsomal assays with UGT1A9 and human urine are shown in Fig. 6. There is clear evidence for the 2 *N*-glucuronides, denoted M1 (RT111) and M2 (RT118), to be produced by UGT1A9 and to be present in extracts of human urine as well. Furthermore, M2 with RT118 appears to be the prominent *N*-glucuronide and accounts for approximately 34% of the total metabolites excreted in human urine within 0 to 72 hours.

4. Discussion

Retigabine is the first selective neuronal potassium channel opener which is currently under advanced clinical development for the treatment of epilepsy. We show UGT1A1 and UGT1A9 to be important in the metabolism of retigabine, which resulted in 2 distinct *N*-glucuronides. In addition, UGT1A1 catalyzed the formation of one of the *N*-glucuronides, whereas UGT1A9 is capable of producing both glucuronides at approximately similar rates, for example, 0.15 vs 0.18 nmol/(min · mg protein).

In the plasma of humans, dogs, and rats, the retigabine *N*2-glucuronide, in which the primary amino group in position 2 represents the site of glucuronidation (Fig. 1, metabolite 2), exceeds by far the amount of the *N*4-glucuronide, which is characterized by the glucuronidation of the secondary amino group in position 4 (Fig. 1, metabolite 1). Therefore, the M2 glucuronide represents the major retigabine metabolite in the plasma of rats, dogs, and humans, whereas the M1 glucuronide could be quantified up to now only in rat bile fluid and in human liver slice and liver microsome in vitro assays [9].

Both *N*-glucuronides, however, could be quantified in human urine samples of healthy male subjects after a single oral 200-mg dose of [^{14}C]-retigabine, and the urinary excretion of the *N*2- and *N*4-glucuronides over 72 hours amounted to 16% and 2% of the administered dose, respectively, thus supporting the overall predominance of the *N*2-glucuronide in vivo.

The retigabine *N*2-glucuronide formation in the plasma of human subjects is extensive and occurs as early as 20 minutes after dosing and achieves maximum plasma concentrations at about 1.5 hours postdose. Notably, the total *N*2-glucuronide exposure (ie, AUC values) considerably exceeded that of retigabine by about 24 ± 10-fold after single dosing and up to 35 ± 5-fold after multiple dosing [16]. However, parallel plasma concentration–time courses of retigabine parent drug and the *N*2-glucuronide point to a constant plasma ratio or equilibrium between retigabine and its *N*2-glucuronide, which was shown to have a remarkably low intersubject variability [16].

As the retigabine *N*-glucuronides were shown to be substrates of β-glucuronidase, it was suggested that the observed in vivo equilibrium between retigabine and retigabine *N*-glucuronides most probably reflects bidirectional glucuronidation and de-glucuronidation processes, which result in the initial formation of a large fraction of retigabine *N*-glucuronides with a subsequent gradual recycling of free parent drug from the *N*-glucuronide pool [16,22]. This hypothesis would be consistent with the finding that the urinary excretion of unchanged retigabine parent drug aggregates to 36% of the administered dose and thus exceeds the urinary excretion of both retigabine *N*-glucuronides approximately 2-fold, although the apparent total plasma exposure of the *N*2-glucuronides exceeds that of retigabine multifold. The concept of a bidirectional N-glucuronidation and de-glucuronidation cycle of retigabine is further supported by similar terminal half-lives of 11.4 and 12.3 hours observed for retigabine and the retigabine *N*2-glucuronide, respectively.

In previous studies [9], metabolites from rat, dog, and human urine were isolated and purified by semipreparative HPLC. Identification of the major *N*-glucuronide by MS/MS

and 2-dimensional 600-MHz proton nuclear magnetic resonance provided conclusive evidence for the primary amino group of retigabine to be the site of glucuronidation. Furthermore, in rat bile and in liver slices of rat and man, McNeilly et al [23] and Hempel et al [9] identified an additional *N*-glucuronide at the secondary amino group of retigabine. As denoted above, an acetyl derivative of retigabine, that is, the carbamic acid ethyl ester moiety was replaced by an acetyl group, was found in plasma and human urine as well. There are obvious species differences in the metabolism of retigabine. Dogs and humans are relatively similar in terms of the metabolic disposal [9], whereas metabolism in rats is somewhat more complex, with more than 10 different metabolites detectable in rat urine, but plasma and bile contain primarily the *N*-glucuronides, too.

Hiller et al [16] investigated retigabine N-glucuronidation and its potential role in enterohepatic circulation. The authors found UGT1A1, UGT1A3, UGT1A4, and UGT1A9 to catalyze the N-glucuronidation of retigabine, albeit at different levels. Significant enterohepatic cycling of the *N*-glucuronides was also suggested. We extend the earlier findings of Hiller et al [16] and report the biosynthesis of one retigabine glucuronide by microsomal preparations from the liver of a patient with Crigler-Najjar type II syndrome and by human kidney microsomes, both of which lacked UGT1A. We suggest that, next to the principal isozymes, other members of the UGT1 and UGT2 gene family may also code for proteins to produce one of the retigabine glucuronides, although participation of isoforms UGT2B7 and UGT2B15 could be excluded, which we had available for incubation assays.

Notably, glucuronidation occurs with many different drugs and chemicals, and glucuronides may be formed with O, N, S, or C atoms. The various aspects of N-glucuronidation in human metabolism of drugs with tertiary amine group as well as species differences in N-glucuronidation were reviewed elsewhere [24].

Several antiepileptic drugs undergo extensive N- and/or O-glucuronidation, and this includes zonisamide, lamotrigine, topiramate, tiagabine, losigamone, and stiripentol [25]. It is intriguing that so many diverse chemicals used in the treatment of epilepsy embark on this particular pathway of metabolic disposal. Noteworthy, lamotrigine as well as retigabine is cleared almost exclusively via phase II metabolism without any indication for any involvement of CYP monoxygenases.

The results from incubations of retigabine with microsomal preparations of human kidney—which lacks UGT1A1—provided evidence that metabolic disposal of this drug is not confined to liver function. Whether other tissues (eg, gut wall) also contribute to the metabolism of retigabine is, as yet, unknown.

In conclusion, we identified several members of the UGT1 family to be involved in the metabolism of retigabine glucuronides. Functional loss of the entire UGT1A gene (Crigler-Najjar syndrome) did not completely block the metabolic disposal of retigabine, which suggests the involvement of other members of the UGT1 and UGT2 gene family. The production of retigabine glucuronides also proceeded with microsomal preparations from the kidney, thus indicating that metabolism is not exclusively confined to liver tissue.

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