Effect of Aging on Glucuronidation of Valproic Acid in Human Liver Microsomes and the Role of UDP-Glucuronosyltransferase UGT1A4, UGT1A8, and UGT1A10

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ABSTRACT:

Valproic acid (VPA) is a widely used anticonvulsant that is also approved for mood disorders, bipolar depression, and migraine. In vivo, valproate is metabolized oxidatively by cytochromes P450 and β -oxidation, as well as conjugatively via glucuronidation. The acyl glucuronide conjugate (valproate-glucuronide or VPAG) is the major urinary metabolite (30–50% of the dose). It has been hypothesized that glucuronidation of antiepileptic drugs is spared over age, despite a known decrease in liver mass. The formation rates of VPAG in a bank of elderly (65 years onward) human liver microsomes (HLMs) were measured by liquid chromatography/tandem mass spectrometry and compared with those in a younger (2–56 years) HLM bank. In vitro kinetic studies with recombinant UDP-glucuronosyltransferases (UGTs) were completed. A 5- to 8-fold variation for the formation of VPAG was observed within the microsomal bank obtained from elderly and younger donors. VPAG

formation ranged from 6.0 to 53.4 nmol/min/mg protein at 1 mM substrate concentration ($n = 36$). The average velocities at 0.25, 0.5, and 1 mM VPA were 7.0, 13.4, and 25.4 nmol/min/mg protein, respectively, in the elderly HLM bank. Rates of VPAG formation were not significantly different in the HLM bank obtained from younger subjects. Intrinsic clearances ($V_{\max}/K_m$) for several cloned, expressed UGTs were determined. UGT1A4, UGT1A8, and UGT1A10 also were found to catalyze the formation of VPAG in vitro. This is the first reported activity of these UGTs toward VPA glucuronidation. UGT2B7 had the highest intrinsic clearance, whereas UGT1A1 demonstrated no activity. In conclusion, our investigation revealed no differences in VPAG formation in younger versus elderly HLMs and revealed three other UGTs that form VPAG in vitro.

Glucuronidation is the conjugation of a glucuronic acid moiety to a range of functional groups—primary, secondary, tertiary, and aromatic amines, carboxylic acids, thiols, hydroxyls, and phenolic functional groups of xenobiotics or endogenous compounds. This conjugative reaction is carried out by UDP-glucuronosyltransferases (UGTs), enzymes expressed on the inner membrane of the endoplasmic reticulum (Dutton, 1966; Mackenzie et al., 1997; Remmel et al., 2008). Although predominantly expressed in the liver, UGTs are diverse in expression as well as function and are categorized into three subfamilies: UGT1A, UGT2A, and UGT2B (Mackenzie et al., 1997; Burchell et al., 1998; Tephly et al., 1998). Four UGT2B isoforms and nine UGT1A isoforms are expressed in humans and catalyze the glucuronidation of a variety of endogenous and exogenous substrates (Burchell et al., 1998; Mackenzie et al., 2000; Tukey and Strassburg,

2001). Glucuronidation is an important metabolic pathway for many drugs or oxidative metabolites. Several antiepileptic drugs (AEDs) including valproic acid (VPA) are excreted extensively as their glucuronides (Levy et al., 2002). VPA is directly glucuronidated to form its ester (acyl) glucuronide that is excreted in the urine, accounting for 30–70% of the administered dose (Dickinson et al., 1989). Additional routes of metabolism include β -oxidation (20–40%) and cytochrome P450-mediated oxidation and desaturation (~10%; Fig. 1). Most enzymes contributing to the glucuronidation of AEDs are known, but intrinsic clearances of UGTs have not been characterized for each AED. Glucuronidation of VPA has been reported to be carried out by UGT1A3, UGT1A6, UGT1A9, UGT2B7, and UGT2B15 (Green and Tephly, 1996; Green et al., 1998; Ethell et al., 2003).

The effect of aging on glucuronidation has not been well studied. Clearance of benzodiazepines such as oxazepam, temazepam, and lorazepam was not substantially altered in younger versus elderly subjects (20–84 years) (Greenblatt et al., 1980; Divoll et al., 1981; Greenblatt et al., 1991a,b). It has been hypothesized that glucuronidation is generally unchanged in elderly population, despite a known decrease in liver mass (Greenblatt et al., 1991a,b). Altered drug clearance in the elderly can be attributed to changes in, e.g., liver mass, liver blood flow, and plasma protein binding (Perucca et al.,

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; AED, antiepileptic drug; VPA, valproic acid; VPAG, valproate glucuronide; HLM, human liver microsome; YHLM, young human liver microsome; EHLM, elderly human liver microsome; LC-MS/MS, liquid chromatography/tandem mass spectrometry; CDI, 1,1'-carbonyl di-imidazole; Cl_{int} , intrinsic clearance.

FIG. 1. Metabolism of VPA. VPA is metabolized to its acyl glucuronide (30–50% of the dose). We found three new UGT isozymes (UGT1A4, UGT1A8, and UGT1A10) that carry out this reaction in vitro. Other pathways of metabolism include mitochondrial β -oxidation and nominal oxidation by cytochrome P450 enzymes.

1984; Wynne et al., 1989; Fattore et al., 2006); however, these factors alone fail to explain the alteration in clearance observed in the elderly (Durnas et al., 1990).

The objective of the current investigation was to study the variability in metabolism of VPA by glucuronidation in a human liver microsomal (HLM) bank. HLMs in this bank were divided into two groups of young (2–56 years: YHLMs, $n = 18$) and elderly (65 years onward: EHLMs, $n = 18$) and screened for glucuronidation activity against VPA. To explore the relative contributions of other predominant UGTs toward VPA glucuronidation, in vitro experiments with Supersomes from the UGT1A and UGT2B subfamilies were also carried out. The present study reveals the variability and effect of age on the glucuronidation of VPA in HLMs. Three additional UGT isozymes were found to possess catalytic activity for VPAG formation in vitro.

Materials and Methods

Chemicals. Formic acid was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). HPLC grade ammonium acetate, magnesium chloride, ethyl acetate, methanol, potassium chloride, dibasic and monobasic potassium phosphate, glycerol, and methyl-*tert*-butyl ether were purchased from Fisher Scientific Co. (Waltham, MA). Trizma buffer, saccharo-1,4-lactone, VPA, tetrasodium pyrophosphate, tetrabutyl ammonium hydroxide 30-hydrate, 1,1'-carbonyl di-imidazole (CDI), glucuronic acid, sodium hydride, triethyl amine, and uridine diphosphoglucuronic acid were purchased from Sigma-Aldrich (St. Louis, MO). UGT Supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, and UGT1A10) were obtained from BD Biosciences (Franklin Lakes, NJ). High-purity acetonitrile for LC-MS/MS was obtained from Honeywell Burdick & Jackson (Muskegon, MI). Alamethicin was purchased from Fluka (Buchs, Switzerland). Nylon membrane filters were obtained from Whatman (Maidstone, UK).

Preparation of Human Liver Microsomes. The HLMs in the microsomal bank were either prepared from transplant-grade donor tissue or resected normal tissue surrounding a liver tumor or purchased commercially from

CellzDirect, Inc. (Durham, NC). The HLMs made in the laboratory were prepared by modification of previously described methods (Vaz et al., 1992; Nelson et al., 2001; Kolwankar et al., 2005). In short, chunks of the frozen liver tissue (~5–10 g) were weighed and $3\times$ (v/w) of 1.15% KCl in 0.01 M potassium phosphate buffer, pH 7.4, was added. The tissue samples were placed on ice until the chunks thawed and homogenized (in a graduated cylinder) with Polytron homogenizer (Brinkmann Instruments, Westbury, NY), and volume was measured. The homogenates were spun at 9500g for 20 min at 4°C (8500 rpm, model J2-MC centrifuge; Beckman Coulter, Fullerton, CA). Supernatants were filtered through gauze, and the total volume was measured. The supernatant (S9 fraction) was divided into ultracentrifuge, which was then centrifuged at 100,000g for 65 min at 4°C (35,000 rpm, model L-90K centrifuge; Beckman Coulter). Microsomes were at the bottom of the tube and were carefully removed from surrounding pasty material (glycogen) and pooled. The microsomal pellet was resuspended in buffer consisting of 1 M sodium pyrophosphate solution, pH 7.4, and homogenized on ice in a Potter-Elvehjem homogenizer and was centrifuged a second time at 100,000g for 65 min at 4°C. The pellet from this spin was resuspended in 0.1 M phosphate buffer containing 1 mM EDTA and 20% glycerol (one third of the cytosolic volume) and was aliquoted into labeled plastic vials to be stored at -80°C after homogenization on ice (Chen et al., 2005). Protein was determined by a bicinchoninic acid dye method (BCA assay; Pierce Chemical, Rockford, IL). Our bank of HLMs was made up of the microsomes prepared as mentioned above as well as some commercially purchased HLMs. The HLMs were divided into two groups of $n = 18$ each, based on the donor's age: YHLMs obtained from younger donors, age 2 to 56 years; and EHLMs obtained from elderly donors, 65 years onward. Pooled HLMs were prepared by combining individual preparations. VPA glucuronidation was measured in all YHLMs and EHLMs at 250, 500, and 1000 μM substrate concentrations. Full kinetic characterization of VPA glucuronidation activity was carried out in five HLMs randomly chosen from each set.

Preparation of Synthetic Standard of VPAG. VPAG standard is not commercially available. However, it was necessary to have VPAG standard for

FIG. 2. Synthesis of VPAG. VPAG standard was chemically synthesized by modification of a method previously used by Becker et al. (1996) to synthesize retinoic acid glucuronide. 1, glucuronic acid; 2, tetrabutyl ammonium glucuronate; 3, VPA; 4, valproyl imidazole; and 5, VPAG. a, tetrabutyl ammonium hydroxide, methanol, room temperature, 24 h. b, VPA, pyridine, 1,1-CDI, room temperature, 2 h. c, sodium hydride, acetic acid, pyridine, room temperature, 6 to 8 h.

carrying out kinetic experiments in HLMs and recombinant UGTs. VPAG standard was synthesized by modification of a previously reported method (Becker et al., 1996). The free acid of VPA was obtained as a precipitant liquid, by treatment of sodium valproate with 10% HCl.

Synthesis of Tetrabutyl Ammonium Glucuronate. Glucuronic acid (2.9 g) was suspended in 25 ml of methanol and stirred for 15 min. Tetrabutyl ammonium hydroxide-30 hydrate (12 g) was then added, and the suspension was stirred for 24 h until a clear solution resulted. The solution was then concentrated on a rotary evaporator at 40°C. Approximately 500 ml of acetone was added until precipitate fell out, and the solution was left in the refrigerator overnight to maximize the yield. The precipitate was filtered and washed with acetone. Tetrabutyl ammonium glucuronate was obtained as a white powder (5.6 g; 85% yield).

Synthesis of VPAG. VPA (2.2 g) was dissolved in 15 ml of pyridine and stirred at room temperature for 5 min. CDI (1.2 g) was added and the mixture was stirred for 2 h to obtain VPA-imidazole as the major product. Tetrabutyl ammonium glucuronate (3.2 g) was added along with 10 ml of pyridine and ~20 mg of sodium hydride, and the reaction was stirred at room temperature for 6 to 8 h. Alternatively, dissolution of tetrabutyl ammonium hydroxide (3.2 g) and 1.1 g of CDI in 25 ml of dichloromethane and 2 ml of triethyl amine afforded instant effervescence and afforded a cleaner subsequent workup. This mixture was stirred at room temperature for 1 h, after which 1.05 g of VPA was added, and the reaction was stirred for 6 to 8 h. The reaction was stopped by careful addition of water, and the solution was made slightly acidic by addition of acetic acid (pH 5.5–6). The synthetic scheme is presented in Fig. 2. The reaction mixture was separated on a semipreparative C-18 Haisil column (100 × 20 mm, 5 μ m; Thermo Fisher Scientific) at a flow rate of 10 ml/min, with UV detection at 210 nm. The gradient consisted of 80% 20 mM acetate buffer, pH 3.0, and 20% acetonitrile at 0.1 min linearly transformed to 50% each, at 22 min, followed by a 6-min re-equilibration step. The HPLC separation revealed two major peaks, one of which was VPA. The peak corresponding to the glucuronide was collected and analyzed on a TSQ triple quadrupole mass spectrometer (Thermo Fisher Scientific). LC-MS/MS analysis revealed a spectrum with major ion of m/z 319.2, undergoing neutral loss of m/z 176 (glucuronic acid) to m/z 143.2 (VPA). Thus, VPAG was the major product formed in the reaction mixture. The peak of interest was collected after multiple injections of the reaction mixture onto the semipreparative column. The collected VPAG fractions were pooled and evaporated to dryness on nitrogen dryer (TurboVap; Zymark, Hopkinton, MA). The residue was reconstituted in minimal water and purified by extraction with ethyl acetate to afford VPAG (110 mg; 12.5% yield).

Glucuronidation Assays. The conditions of analysis for incubations with HLMs and cloned, expressed UGTs were identical except for the protein concentrations. Each HLM suspension was diluted in water to obtain a final protein concentration of 0.29 mg/ml. Incubation for each substrate concentration was carried out in triplicate. For in vitro incubations, the HLMs obtained from elderly and younger donors were mixed on a vortex mixer with ala-

methicin (~50 mg/mg protein) and preincubated on ice for 15 min. Trizma buffer (0.1 M; pH 7.4) at 37°C was then added, followed by the addition of magnesium chloride (5 mM), saccharo-1,4-lactone (5 mM), and VPA (final concentration range, 100 μ M–20 mM). This mixture was preincubated at 37°C for 2 min after vortex mixing for 30 s. The reaction was started by addition of freshly prepared uridine diphosphoglucuronic acid (3 mM) to yield a final incubation volume of 0.2 ml. The final concentration of total organic in the incubation did not exceed 1%. The reaction was carried out for 40 min at 37°C and 30 rpm in a water bath (model BS-11; Lab Companion, Seoul, Korea). An equal volume of chilled 2% acetic acid in methanol was used to stop the reaction, followed by centrifugation at 13,000 rpm for 5 min, through 0.2- μ m nylon spin filters. The protein content for recombinant UGT incubations was 0.125 mg/ml. Both the protein concentration and incubation time were determined to be in the linear range in preliminary experiments (Chen et al., 2005).

LC-MS/MS Assay for VPAG. Valproate glucuronide was highly sensitive to pH and heat; hence an LC-MS/MS method was developed to provide the sensitivity required for the process (Chen et al., 2005). The VPAG standard (10 mg/ml) was infused at a syringe flow rate of 12 μ l/min into the source of TSQ triple quadrupole mass spectrometer (Thermo Fisher Scientific), under negative ion mode. The formation of the parent ion VPAG ($[M - 1]^-$; m/z 319.2) was optimal at the spray voltage of -3.25 kV and collision energy of 33 eV. The parent ion was fragmented with argon as the collision gas, to yield a major daughter ion of m/z 143.2. (Fig. 3). Other conditions were as follows: capillary temperature, 110°C; collision pressure, 1.0 mTorr; and sheath gas pressure, 39 ml/min.

With the above-mentioned instrument parameters, samples were assayed for the presence of VPAG and on TSQ triple quadrupole mass spectrometer equipped with a capillary HPLC system (1100 series; Agilent Technologies, Santa Clara, CA). The separation was carried out by injecting an aliquot (2 μ l) on a β -Basic C-18 column (150 × 1 mm, 3 μ m; Thermo Fisher Scientific) at room temperature. A gradient composed of 30 to 50% acetonitrile in 20 mM ammonium acetate buffer, pH 3.77, over a period of 6 min was used to separate VPA from VPAG. It was held at 50% acetonitrile for 4 more minutes, followed by a 4-min re-equilibration step. The flow rate for the analysis was 10 μ l/min. Limit of quantitation was 0.1 nM, as determined from separate injections of VPAG standards. For detection, data acquisition was achieved on Xcalibur software (Thermo Fisher Scientific). Standard curves for VPAG were developed with VPAG concentrations of 3 μ M to 3 mM and showed an $R^2 \geq 0.999$. The coefficient of variation was <10% at all concentrations of VPAG. Figure 3 shows the mass spectrum and chromatogram for VPAG in a microsomal incubation. Kinetic analysis was performed with SigmaPlot (Systat Software, Inc., San Jose, CA). Statistical analysis (standard Student's *t* test) was performed on the sample sets after determining that the sample populations had identical standard deviations (Bartlett statistic, $p > 0.1$) and displayed a Gaussian distribution (Kolmogorov-Smirnov test, $p > 0.1$).

FIG. 3. A, LC-MS/MS of VPAG. Mass spectrum of the purified VPAG standard. The purified VPAG standard showed a SRM transition from m/z 319.20 to m/z 143.20. B, selected reaction monitoring chromatogram of VPAG by LC-MS/MS assay. VPAG was quantified by monitoring the optimized selected reaction monitoring of m/z 319.0 to m/z 143.20. HPLC gradient was used for the separation of VPAG on a C-18 column.

Results

Results from Linearity Studies. Linearity studies were initially conducted to determine the optimum time and protein concentration for carrying out the incubations. The velocity of VPAG formation

seemed to be linear of over a period of 0 to 60 min and over a protein concentration range of 0.1 to 0.5 mg/ml, with the use of alamethicin. Based on these results, a protein concentration of 0.29 mg/ml and a time of 40 min were chosen for the incubation reaction. Figure 3

FIG. 4. Variation in VPA glucuronidation across elderly HLM bank (A) and younger HLM bank (B). The results are indicated as means of triplicate measurements $\pm$ S.D. The incubations were carried out at three different VPA concentrations: 250 μ M ($\square$), 500 μ M ($\blacksquare$), and 1000 μ M ($\square$).

shows the mass spectrum and chromatogram for VPAG in a microsomal incubation.

In Vitro Glucuronidation in Elderly Liver Microsomal Bank. Approximately 8-fold variation for the formation of VPAG was observed within the microsomal bank (Fig. 4). The range for the velocity of VPAG formation extended from 6.8 to 53.4 nmol/min/mg protein at 1 mM substrate concentration. The average velocity at 1 mM VPA concentration was 25.4 ± 11 nmol/min/mg protein, whereas that at 0.25 and 0.5 mM substrate concentration was 7.0 ± 5 and 13.4 ± 6 nmol/min/mg protein, respectively. The variability was same for all three concentrations of VPA.

In Vitro Glucuronidation in Younger Liver Microsomal Bank. Likewise, within the bank of 18 HLMs obtained from younger donors (2–56 years), a 7-fold variation for the formation of VPAG was observed (Fig. 4). The range for the velocity of VPAG formation extended from 6.0 to 45.0 nmol/min/mg protein at 1 mM substrate concentration. The average velocity at the same substrate concentration was 24.5 ± 9 nmol/min/mg protein, whereas that at 0.25 and 0.5 mM substrate concentration was 6.8 ± 3 and 14.0 ± 6 nmol/min/mg protein, respectively. There was no statistically significant difference in the mean velocities for VPAG formation between the two HLM banks (Student's *t* test, $p > 0.05$).

VPA Glucuronidation in HLMs and Cloned, Expressed UGTs. Glucuronidation of VPA was determined in five HLMs from the younger and elderly donor bank each. The average $V_{\max}$, K_m , and intrinsic clearance values were not significantly different in the five EHLs compared with the five YHLs. The mean intrinsic clearance in the five EHLs was 52.98 ± 12.2 μ l/min/mg, whereas that in the five YHLs was found to be 45.2 ± 10.5 μ l/min/mg. Full kinetic studies conducted in pooled HLMs yielded a $V_{\max}$ value of

32.6 ± 0.6 nmol/min/mg protein and a K_m value of 2.4 ± 0.4 mM (data not shown).

Intrinsic clearances ($V_{\max}/K_m$) for several cloned, expressed UGTs (Supersomes) were determined (Table 2). UGT1A4, UGT1A8, and UGT1A10 catalyzed the formation of VPAG, with intrinsic clearances of 0.13, 0.14, and 0.16 μ l/min/mg protein, respectively (Fig. 5). UGT1A3 had an intrinsic clearance of 0.09 μ l/min/mg protein; the lowest of all the screened UGTs. Our results also showed that UGT2B7 had the highest intrinsic clearance 0.86 μ l/min/mg protein, with a $V_{\max}$ value of 1.2 ± 0.15 nmol/min/mg protein, whereas no activity was observed with UGT1A1. Regarding response, the goodness of fit was determined by sum of least-squares method. Michaelis-Menten kinetics seemed to best fit the data. Eadie-Hofstee plots did not result in further information or conclusions.

Discussion

Synthesis of VPAG. Synthesis of acyl glucuronides is difficult because of their low hydrolytic stability, propensity to undergo rearrangement, and a need for a series of protection and deprotection steps in their synthesis (Stachulski and Jenkins, 1998). Alternatively, they can be biosynthesized, but the resultant yields are low and come at the expense of microsomal use (Panfil et al., 1992; Becker et al., 1996). Synthesis of acyl glucuronides with the help of a variety of protecting reagents has been reported previously (Kaspersen and Van Boeckel, 1987; Stachulski and Jenkins, 1998), but all methods are time-consuming and require multiple steps to obtain a benzyl-protected sugar. We applied and further modified a method (Fig. 2) reported previously by Barua and colleagues for the synthesis of retinoic acid glucuronide, to synthesize VPAG (Becker et al., 1996; Chen et al., 2005).

FIG. 5. Michaelis-Menten kinetics for VPA glucuronidation in UGT1A4, UGT1A8, and UGT1A10. UGT1A4, UGT1A8, and UGT1A10 showed Michaelis-Menten type kinetics for glucuronidation of VPA. These UGT isozymes have not been reported previously to catalyze the glucuronidation of VPA in vitro.

We used triethyl amine in the presence of dichloromethane as opposed to pyridine, to initiate the reaction. The resultant reaction mixture was clean and easier to concentrate via a rotary evaporator. However, even without the use of pyridine, visualization of spots on the thin layer chromatography plates was difficult as the VPA-imidazole (intermediate) broke down on silica. Formation of this intermediate could only be monitored by evolution of carbon dioxide during its formation. The reaction reported by Barua and colleagues had low yields initially (25%) (Becker et al., 1996) and higher yields following optimization (68%) (Becker et al., 1996). With the modification of this method, we obtained yields up to 18%. However, the method was not optimized to obtain higher yields. The resulting VPA glucuronide was purified by semipreparative HPLC on a C-18 column.

VPAG LC-MS/MS Assay. In the LC-MS/MS assay as described above, VPAG eluted at 4.8 min. The percentage of organic (acetonitrile) was then increased rapidly to elute out the substrate VPA. Acyl glucuronides have been known to be sensitive to pH changes, and can undergo internal rearrangement at neutral or basic pH (Dickinson et al., 1984). We found that the VPAG could not be analyzed by either gas chromatography or gas chromatography-mass spectrometry because of its low volatility and instability at high temperatures. HPLC-UV analysis of the glucuronide had sensitivity limitations due to lack of any chromophore in the molecular structure. Mass spectrometry conditions such as capillary temperature, spray voltage, nitrogen flow, and quadrupole bias were optimized to obtain maximal sensitivity for the negative ion neutral loss assay.

Acyl glucuronides such as VPAG have been shown to undergo pH-dependent rearrangements (Dickinson et al., 1984, 1985). Although the incubation mixture was analyzed within a short time after carrying out the incubations, tailing of the VPAG peaks was observed. Such tailing of VPAG peaks could have been caused by internal rearrangement of the VPAG. To prevent rearrangement, the incubation reaction was stopped by addition of chilled 2% acetic acid in methanol. This ensured that the glucuronic acid moiety of VPAG was protonated and less susceptible to internal rearrangement. A full scan spectrum and a chromatogram of VPAG are shown in Fig. 3.

Comparative Metabolism and Kinetics in Elderly and Younger HLMs. There was approximately 8-fold intrabank variability (Fig. 4), which is partially responsible for the interindividual variability in VPAG formation in vivo. Our results indicated that VPAG formation rates in HLMs from younger and elderly donors were not significantly different (standard *t* test, $p > 0.5$). Assuming that the intrinsic clearance of AEDs such as VPA may be spared in elderly subjects, a decrease in liver mass with no change in enzyme activity would result in lower total body clearance for a low-clearance drug such as VPA. Based on pharmacokinetic studies with benzodiazepines, Greenblatt and coworkers suggested that total body clearance of drugs that are primarily glucuronidated is not affected by aging. (Greenblatt et al., 1980, 1981a,b, 1991a,b; Divoll et al., 1981; Durnas et al., 1990). Our studies with valproate (a general substrate catalyzed by UGT2B7 and several other enzymes) indicate that VPA total body clearance would decline with the age-related decrease in liver mass, assuming that intrinsic clearance is not altered with aging. However, the microsomes that constituted the HLM bank were obtained from a range of sources, and they were prepared by different methods and stored in different cryopreservation buffer solutions. It is unclear at this point how these factors might affect the functional activity of HLMs.

Full kinetic experiments were carried out in five younger (YHLMs) and five elderly (EHLMs) HLMs as well as pooled HLMs to obtain a measure of VPAG formation in vitro (Table 1). Data collected for each of the microsomal samples (incubations at six concentrations and controls, each in triplicate) were fit to Michaelis-Menten model by

TABLE 1

*Michealis-Menten kinetics in five HLMs from elderly and younger donor bank*Values are represented as means of triplicate measurements $\pm$ S.D.

HLM No.	$V_{\max}$	K_m	$Cl_{\text{int}} = V_{\max}/K_m$
	nmol/min/mg	mM	$\mu\text{L}/\text{min}/\text{mg}$
EHL M 1	44 $\pm$ 2.8	0.8 $\pm$ 0.2	55
EHL M 2	64 $\pm$ 3.0	1.1 $\pm$ 0.4	58.2
EHL M 3	32 $\pm$ 2.7	0.6 $\pm$ 0.3	53.3
EHL M 4	82 $\pm$ 2.8	2.5 $\pm$ 0.6	32.8
EHL M 5	59 $\pm$ 0.9	0.9 $\pm$ 1.0	65.6
Avg.	56.2 $\pm$ 19.2	1.2 $\pm$ 0.8	52.98 $\pm$ 12.2
YHL M 1	37 $\pm$ 2.4	0.7 $\pm$ 0.3	52.9
YHL M 2	45 $\pm$ 2.8	0.8 $\pm$ 0.2	56.3
YHL M 3	82 $\pm$ 3.5	2.4 $\pm$ 0.5	34.2
YHL M 4	85 $\pm$ 1.4	2.5 $\pm$ 0.2	34
YHL M 5	34 $\pm$ 2.0	0.7 $\pm$ 0.6	48.6
Avg.	56.62 $\pm$ 24.9	1.4 $\pm$ 0.9	45.2 $\pm$ 10.5
Pooled HLMs	32.6 $\pm$ 0.6	2.4 $\pm$ 0.4	13.9

nonlinear regression. A range of $V_{\max}$ (32–85 nmol/min/mg protein) and K_m (0.6–2.5 mM) values was observed across the samples. Similar studies conducted in pooled HLMs yielded a $V_{\max}$ value of 32.6 $\pm$ 0.6 nmol/min/mg protein and a K_m value of 2.4 $\pm$ 0.4 mM (data not shown). No significant differences were observed in $V_{\max}$ and K_m values from elderly and younger population (standard *t* test, $p > 0.5$). In seven of the 10 livers, the K_m values ranged from 0.7 to 1.1 mM, a concentration that is similar to the K_m concentration for cloned, expressed UGT2B7, the enzyme with the highest intrinsic clearance. In three other livers, the K_m value was approximately 2.5 mM, a value that is similar to other UGT enzymes. These results could represent the expected interindividual variability in VPA metabolism and UGT expression in patients. The average intrinsic clearance values were not significantly different in the five EHLMs compared with the five YHLMs.

UGTs Involved in Metabolism of VPA. Intrinsic clearances ($V_{\max}/K_m$) for several cloned, expressed UGT Supersomes were determined to screen the UGTs from UGT1A and UGT2B families for their activity for VPA glucuronidation. It was found that UGT1A4, UGT1A8, and UGT1A10 catalyzed the formation of VPAG in vitro, and this to our knowledge is the first reported instance for the activity of these UGTs toward VPA glucuronidation (Fig. 5). Studies with UGT1A3, UGT1A9, and UGT2B7 resulted in similar VPA intrinsic clearances to those reported in the literature (Ethell et al., 2003). Our results also indicated that UGT2B7 had the highest intrinsic clearance compared with UGT1A3, UGT1A9, and UGT1A4, although relative expression levels in liver for these enzymes is not known. UGT1A8 and UGT1A10 may be important contributors to intestinal glucuronidation in vivo because they had the lowest K_m and highest $V_{\max}$ values of the tested enzymes. UGT1A1 demonstrated no activity for VPA glucuronidation.

The intrinsic clearance values reported in Table 2 with cloned, expressed enzymes have not been corrected for the relative contribution of each of the newly found UGTs in vivo. The lack of selective antibodies for each major UGT isoform has made it difficult to obtain the relative expression of various UGTs in liver and intestine. It should also be noted that based on these in vitro experiments, multiple UGTs—UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, and UGT2B7—may be involved in the metabolism of VPA. UGT1A8 and UGT1A10 are primarily intestinal forms. Hence, VPA seems to be a general substrate for glucuronidation.

An approximate 8-fold variation was observed for glucuronidation of VPA within the bank of elderly as well as younger HLMs. No significant difference was observed between microsomes from elderly

TABLE 2

*UGTs involved in glucuronidation of VPA in vitro*Glucuronidation activity was measured in eight UGT Supersomes at different concentrations of VPA. Values are represented as means of triplicate measurements $\pm$ S.D.

UGT Enzyme	$V_{\max}$	K_m	Cl_{int}
	nmol/min/mg	mM	$\mu\text{L}/\text{min}/\text{mg}$
UGT1A1	N.A.	N.A.	
UGT1A3	0.6 $\pm$ 0.3	6.4 $\pm$ 0.76	0.09
UGT1A4	0.4 $\pm$ 0.1	3.1 $\pm$ 0.32	0.13
UGT1A6	0.70 $\pm$ 0.44 ^a	3.2 $\pm$ 0.53 ^a	0.22 ^a
UGT1A8	0.87 $\pm$ 0.5	5.9 $\pm$ 0.76	0.14
UGT1A9	0.96 $\pm$ 0.5	6.3 $\pm$ 0.75	0.15
	0.98 $\pm$ 0.06 ^a	5.2 $\pm$ 0.84 ^a	0.19 ^a
UGT1A10	0.88 $\pm$ 0.3	5.5 $\pm$ 0.39	0.16
UGT2B7	1.2 $\pm$ 0.15	1.4 $\pm$ 0.09	0.86
	0.66 $\pm$ 0.44 ^a	2.1 $\pm$ 0.35 ^a	0.31 ^a

N.A., no activity.

^a Denotes literature-reported values (Ethell et al., 2003).

and those obtained from younger donors. These results support the hypothesis that metabolism of VPA is spared in elderly subjects. However, a known decrease in liver mass could result in lower in vivo clearance of AEDs in elderly population. These results must be considered along with the information from in vivo metabolism studies to comprehend the overall consequence of aging on VPA metabolism. UGT1A4, UGT1A8, and UGT1A10 contributed to formation of VPAG in vitro, with a Cl_{int} value of 0.13, 0.15, and 0.16 $\mu\text{L}/\text{min}/\text{mg}$, respectively. To our knowledge this is the first reported instance for activity of these UGTs toward VPA glucuronidation. The intrinsic clearance for UGT1A4 (1A8 and 1A10 are intestinal forms) was much lower than for UGT2B7, although relative expression data are not available (may be possible via quantitative proteomics approaches in the near future).

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