In Vitro Characterization of Hepatic Flavopiridol Metabolism Using Human Liver Microsomes and Recombinant UGT Enzymes

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Received December 18, 2001; accepted January 4, 2002

Purpose. To assess the contribution of drug metabolism to the variability on flavopiridol glucuronidation observed in cancer patients, and to determine the ability of all known human UDP-glucuronosyltransferase (UGT) isoforms to glucuronidate flavopiridol.

Methods. Inter-individual variation in flavopiridol glucuronidation was determined by HPLC using hepatic microsomes from 62 normal liver donors. Identification of enzymes capable of glucuronidating flavopiridol was determined by LC/MS using human embryonic kidney 293 (HEK293) cells stably expressing all sixteen known human UGTs.

Results. The major product of the flavopiridol glucuronidation reaction in human liver microsomes was FLAVO-7-G. High variability (coefficient of variation = 49%) was observed in the glucuronidation of flavopiridol by human liver microsomes. *In vitro* formation of FLAVO-7-G and FLAVO-5-G was mainly catalyzed by UGT1A9 and UGT1A4, respectively. Similar catalytic efficiencies (V_{max}/K_m) were observed for human liver microsomes (1.6 μl/min/mg) and UGT1A9 (1.5 μl/min/mg).

Conclusions. UGT1A9 is the major UGT involved in the hepatic glucuronidation of flavopiridol in humans. The data suggests that hepatic glucuronidation may be a major determinant of the variable systemic glucuronidation of flavopiridol in cancer patients. The large variability in flavopiridol glucuronidation may be due to differences in liver metabolism among individuals, as a result of genetic differences in UGT1A9.

KEY WORDS: flavopiridol; glucuronidation; UGT1A9; metabolism; liver.

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; FLAVO-7-G, flavopiridol-7-glucuronide; FLAVO-5-G, flavopiridol-5-glucuronide; HEK293, human embryonic kidney 293; LC/MS, liquid chromatography/mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; propofol-G, propofol glucuronide.

INTRODUCTION

Flavopiridol (NSC 649890) (Fig. 1) is a synthetic flavone derivative currently undergoing Phase II clinical development as an anticancer agent. Its mechanism of action is direct inhibition of cyclin-dependent kinases (1,2). The major dose-limiting toxicity of flavopiridol is secretory diarrhea (2,3). Flavopiridol is metabolized in rats and humans via glucuronidation (4,5).

Glucuronidation by uridine diphosphate glucuronosyltransferase (UGT) enzymes is a major drug metabolic pathway in humans. This conjugation reaction results in the formation of water-soluble products that are readily excreted into the bile or urine (6,7). The UGT enzymes are broadly classified into 2 distinct families, UGT1 and UGT2, based on similarities between their primary amino acid sequences (8). Sixteen human UGT enzymes have been cloned: nine functional UGT1A isoforms (UGT1A1 and UGT1A3-10) and seven UGT2B isoforms (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28) (9,10).

Studies of flavopiridol metabolism in human liver microsomes showed the formation of two monoglucuronides (11). These were identified as 7-O-β-glucopyranuronosyl-flavopiridol (FLAVO-7-G) and 5-O-β-glucopyranuronosyl-flavopiridol (FLAVO-5-G) (Fig. 1). FLAVO-7-G was the main product of the flavopiridol glucuronidation reaction. Its formation rate was 50 times higher than that of FLAVO-5-G. Screening of 9 out of the 16 existing human UGT enzymes showed that FLAVO-7-G and FLAVO-5-G were formed mainly by UGT1A9 and UGT1A1, respectively (11).

Upon administration of flavopiridol to metastatic renal cancer patients, Innocenti *et al.* (2000) observed high inter-individual variation (coefficient of variation 72–99%) in the capacity to glucuronidate flavopiridol. Glucuronidation of flavopiridol appeared to be bimodal, and subjects displaying extensive glucuronidation seemed to be protected from suffering diarrhea (5). The aims of this study were 1) to assess the contribution of hepatic metabolism to the variability on flavopiridol glucuronidation observed in cancer patients, and 2) to determine the flavopiridol glucuronidating activity of all known UGT isoforms belonging to both UGT1A and UGT2B families. Our experiments included 7 UGT isoforms that had not been previously screened for their ability to glucuronidate flavopiridol.

MATERIALS AND METHODS

Chemicals and Reagents

Flavopiridol was kindly provided by Drs. Edward A. Sausville and Robert J. Schultz from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, Maryland). All other chemicals and reagents used were of the highest grade and commercially available.

Human Liver Microsome Preparations

Normal human liver specimens were obtained with approval of Institutional Review Boards of the institutions involved through the Liver Tissue Procurement and Distribution System (National Institutes of Diabetes, Digestive and Kidney Diseases/NIDDK NO1-DK6-2274). Microsomes were

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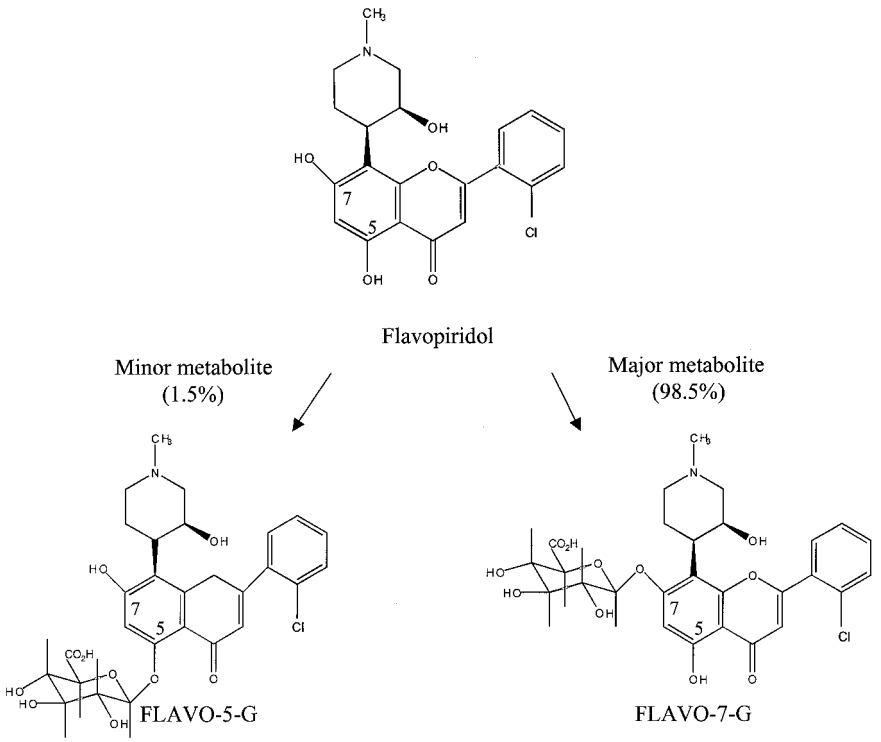

Fig. 1. Glucuronidation of flavopiridol by human liver microsomes. FLAVO-7-G and FLAVO-5-G account for 98.5% and 1.5% of the glucuronidation of flavopiridol, respectively.

prepared from these livers using differential centrifugation methods (12). Total protein content was determined by the Bradford method (13) using bovine serum albumin as standard.

HEK293 Cell Systems Expressing UGT1A and UGT2B

For the analysis of UGT expression and enzymatic activities, membrane fractions from human embryonic kidney 293 (HEK293) cells with stable expression of UGT1A and UGT2B enzymes were prepared as previously described (14). Relative UGT expression levels were determined by a semi-quantitative immunoblot analysis method. For quantification of the UGT1A proteins, we used the anti-human UGT1A common carboxyl-terminus region (amino acids 312 to 531) antiserum RC-71, as previously reported (15). UGT2B protein levels were quantified using the anti-human UGT2B antibody EL-93, as previously described (16). Immunocomplexes were visualized using an enhance chemiluminescence ECL™ kit (ECL™, Amersham, Arlington Heights, Illinois), exposed on hyperfilm for 30 s (Eastman Kodak Co., Rochester, New York), and quantified by BioImage Visage 110s (Genomic Solution Inc., Ann Arbor, Michigan). Linearity in the detection signal was previously demonstrated by parallel analysis of serially diluted UGT enzymes.

Screening of Different UGT Isoforms and Human Liver Microsomes for FLAVO Glucuronidation Activity

A sensitive assay using liquid chromatography tandem mass spectrometry (LC/MS) was developed to determine the activities of UGT enzymes (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28) and human liver microsomes toward flavopiridol. Incubations (100 μL) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 μg/ml phosphatidylcholine, 8.5 mM saccharolactone, 2 mM UDPGA, 0.4–0.6 mg/ml membrane protein and 200 μM flavopiridol. Reaction mixtures were incubated with all UGT1A and UGT2B isoenzymes for 4 h at 37°C, except for UGT2B17, for which an incubation time of 30 min was used due to its lability. The reaction was linear under these conditions. Higher incubation times (up to 16 h) did not change the substrate specificity of the UGT enzymes (data not shown). UGT expression levels were determined by Western blot analysis as described above, and used to convert glucuronidation rates into values normalized to the UGT expression (relative glucuronidation rates).

Reaction mixtures were analyzed by LC/MS using a Zorbax C_{18} column (5 μm, 7.5 × 4.6 mm; Agilent Technologies, Palo Alto, California). The mobile phase consisted of 1 mM ammonium formate in water (A) and 1 mM ammonium formate in methanol (B). The following gradient was applied at a flow rate of 0.8 ml/min: 0–5.5 min from 75% A and 25% B to 48% A and 52% B; 5.5–7.5 min from 48% A and 52% B to 5% A and 95% B; 7.5–8.0 min, hold at 5% A and 95% B; 8.0–10.0 min from 5% A and 95% B to 75% A and 25% B. The effluent from the high performance liquid chromatography (HPLC) system (Alliance Model 2690, Waters Corporation, Milford, Massachusetts) was connected directly to an LCQ™ ion trap mass spectrometer (Thermo Finnigan, San Jose, California) with an electrospray source operated in the positive ion mode. The data was acquired in two events. First, the mass spectrometer was operated in full scan MS and the flavopiridol ion (402 m/z) was monitored (Fig. 2). Second, full scan MS/MS was used to obtain the ion corresponding to the

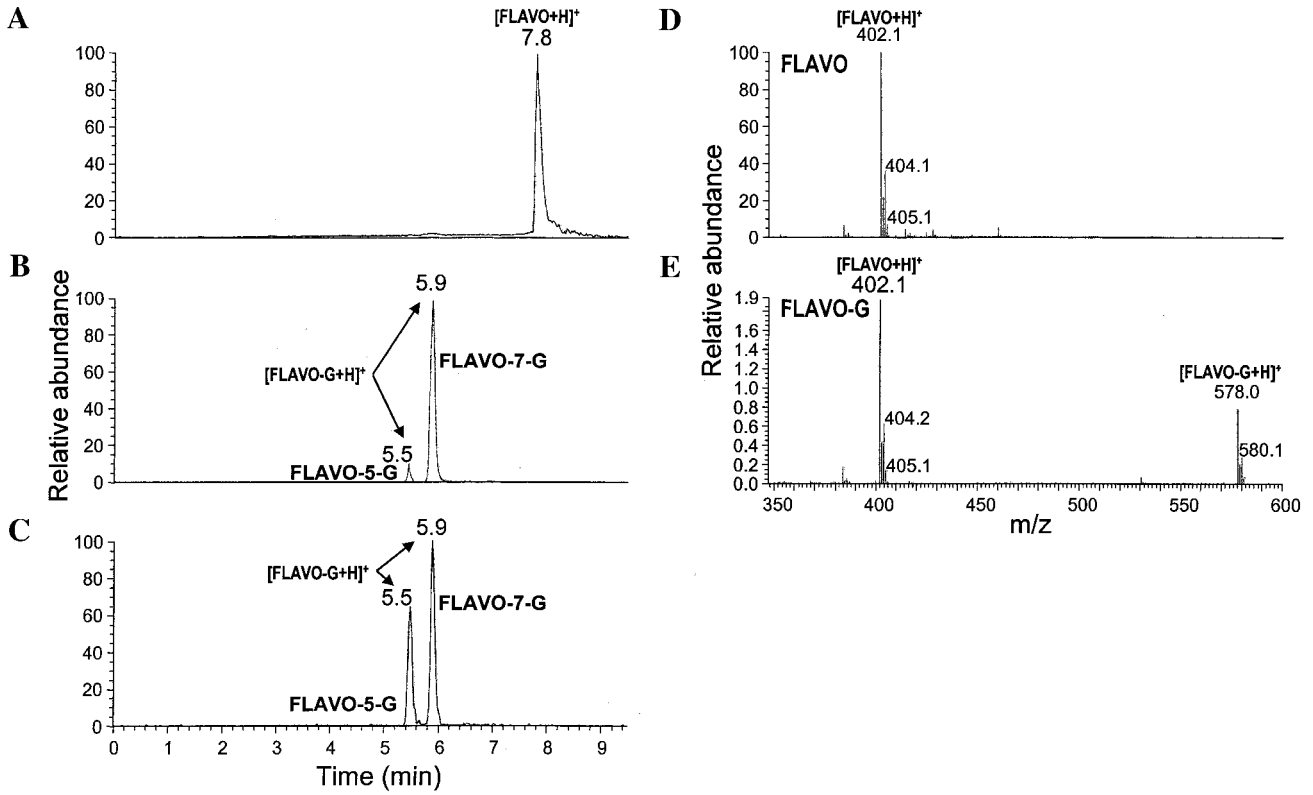

Fig. 2. Characterization of formation of flavopiridol glucuronides using LC/MS. Panels A, B and C correspond to the LC/MS spectra of flavopiridol (A), and FLAVO-5-G and FLAVO-7-G (B and C) after incubation with human liver microsomes (B) and UGT1A4 HEK-293 membranes (C). Molecular weights were determined by mass spectrometry for flavopiridol (D), and FLAVO-5-G and FLAVO-7-G (E). Analysis of a flavopiridol standard showed a positive ion mass spectra similar to panel A, confirming that the peak eluting at 7.8 min with m/z 402.1 corresponds to $[FLAVO+H]^+$. Analysis of incubation mixtures revealed two additional peaks with retention times of 5.9 and 5.5 min corresponding to FLAVO-7-G and FLAVO-5-G, respectively. Both metabolites were observed at m/z 578.0 corresponding to the flavopiridol glucuronides $[FLAVO-G+H]^+$ (Panel E).

flavopiridol glucuronides (578 m/z) (Fig. 2). Retention times for FLAVO-5-G, FLAVO-7-G and flavopiridol were 5.5, 5.9 and 7.8 min, respectively (Fig. 2).

Determination of Kinetic Parameters

Kinetic analyses were performed using an assay with labeled co-substrate UDPGA as previously described (14–16). This method measured the total formation of flavopiridol glucuronides (FLAVO-7-G + FLAVO-5-G). Incubations (100 μL) were performed at 37°C for 90 min with a range of flavopiridol concentrations (0.01-2mM), and microsomes prepared from both human liver and UGT1A9-HEK293 cells (2 mg/ml). Samples were analyzed by thin layer chromatography (TLC).

Inter-Individual Variability in *in Vitro* FLAVO Glucuronidation

Due to the clinical relevance of the flavopiridol glucuronidation reaction, we wanted to characterize the inter-individual variability in a large set of human liver microsomes. Incubations (250 μL) contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 3 mM UDPGA, 3 mg/ml microsomal protein and 500 μM flavopiridol. After incubation for 3 h in a 37°C shaking water bath, the reaction was terminated by adding 1 ml of acetonitrile. Precipitated proteins were removed by centrifugation (15 min, 10,000 *g*, 4°C). The supernatant was spiked with 10 μg of internal standard (flavone), and evaporated to dryness using nitrogen gas. Negative control experiments were performed simultaneously by omitting UDPGA. The samples were reconstituted with 250 μL of the initial mobile phase. Aliquots (125 μL) were analyzed by HPLC (Hitachi Instruments, Inc., San Jose, California) with UV detection (265 nm). Reversed-phase chromatography was carried out using a μBondapak™ Phenyl column (10 μm, 3.9 × 300 mm; Waters Corporation, Milford, Massachusetts) and a μBondapak™ Phenyl guard-pak insert (Waters Corporation, Milford, Massachusetts). The mobile phase consisted of 50 mM ammonium acetate containing 0.1% (v/v) triethylamine (pH 4.15) (A) and acetonitrile (B). Elution was performed at a flow rate of 2 ml/min using the following gradient: 0–5 min, 80% A and 20% B; 5–15 min, 10% A and 90% B; 15–30 min, 80% A and 20% B. Under these conditions, FLAVO-7-G, FLAVO-5-G, flavopiridol and flavone elute at 6.5, 7.5, 11.5, and 13.5 min, respectively.

Formation of only one flavopiridol glucuronide was detected using the conditions described above. Formation of a glucuronide was confirmed by hydrolysis with β-glucuronidase enzyme (*E. Coli* type IX-A, Sigma-Aldrich Co., St. Louis, Missouri) and UV spectroscopy. The incubation reaction was performed as described above. After centrifugation, the supernatant was treated for 24 h with 2,500 units of

β-glucuronidase dissolved in 500 μL of 0.1 M sodium phosphate buffer (pH 6.4). HPLC analysis revealed the disappearance of the glucuronide peak. Analysis of a control sample incubated with buffer alone showed intact glucuronide. To identify the flavopiridol glucuronide, its peak was collected and analyzed by UV spectroscopy. Spectrophotometric scans obtained after the addition of aluminum chloride (500 μM) identified the glucuronide as FLAVO-7-G (data not shown). FLAVO-7-G formation was expressed as the ratio of FLAVO-7-G/flavone peak heights.

The intra-assay variability was determined by performing eight incubations on the same day using a pool of human liver microsomes. The inter-assay variability was evaluated by incubating a pool of human liver microsomes in triplicate on 3 different days. The inter- and intra-assay variabilities were within 8%.

Correlation Studies in Human Liver Microsomes

To test the level of correlation between the glucuronidation of flavopiridol and propofol, a substrate of UGT1A9 (17), we analyzed propofol glucuronidation rates in 30 human liver microsomes. Incubations (250 μL) contained 0.2 M Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM UDPGA, 1 mg/ml human liver microsomal protein and 0.2 mM propofol. The reaction was stopped after 30 min at 37°C by adding 200 μL of methanol. After protein removal by centrifugation (15 min, 10,000 *g*, 4°C), the supernatant was combined with 11 ng of internal standard (*p*-fluorophenol). Aliquots of 100 μL were analyzed by HPLC (Hitachi Instruments, Inc., San Jose, California) with fluorescence detection ($\lambda_{excitation} = 276$ nm, $\lambda_{emission} = 310$ nm). Negative control experiments were performed simultaneously by omitting UDPGA. Elution was performed using a μBondapak™ C_{18} column (5 μm, 3.9 × 300 mm; Waters Corporation, Milford, Massachusetts) and a μBondapak™ C_{18} guard-pak insert (Waters Corporation, Milford, Massachusetts). A mix of acetonitrile, deionized water and acetic acid (35/65/0.1, v/v/v) was used as mobile phase. The flow rate was 2 ml/min from 0–15 min, and 2.5 ml/min from 15–60 min. Under these conditions, *p*-fluorophenol, propofol glucuronide (propofol-G) and propofol eluted at 4.0, 6.2, and 47.0 min, respectively. Since propofol-G was not available in our laboratory, we expressed propofol glucuronidation activity as the ratio of propofol-G/*p*-fluorophenol peak heights.

The intra-assay variability was determined by performing nine incubations on the same day using a pool of human liver microsomes. The inter-assay variability was evaluated by incubating a pool of human liver microsomes in triplicate on three different days. The inter- and intra-assay variabilities were within 7%.

Data Analysis and Statistics

Results were expressed as mean ± standard deviation (SD) of a single experiment performed in triplicate, unless otherwise specified. Apparent K_m and V_{max} were calculated using a Lineweaver-Burk plot. The Spearman correlation coefficient was used to test the level of correlation between the glucuronidation of flavopiridol and propofol. The threshold value for statistical significance was set at 0.05. Data analysis and statistics were done using GraphPad software (GraphPad Software Inc., San Diego, California).

RESULTS

Identification of FLAVO-7-G

Identification of FLAVO-7-G as the major product of the flavopiridol glucuronidation reaction in human liver microsomes was performed using UV spectroscopy. A previous study reported that a complex between Al^{3+} and a free hydroxyl group in position 5 causes a bathochromic shift in the absorption bands of FLAVO-7-G (4). We observed this shift after combining aluminum chloride and the main flavopiridol glucuronide formed by human liver microsomes, and concluded that it was formed by the conjugation of UDPGA with the alcohol group in the C-7 of FLAVO (7-O-β-glucopyranuronosyl-flavopiridol) (Fig. 1).

In Vitro Glucuronidation of FLAVO By cDNA Expressed UGT Isoforms and Human Liver Microsomes

Sixteen individual human UGT isoforms expressed in HEK293 cells and pooled human liver microsomes were screened for flavopiridol activity. Only members of the UGT1A family were involved in the glucuronidation of flavopiridol (Table I). The formation of FLAVO-7-G was mainly catalyzed by UGT1A9. Minor formation of FLAVO-7-G (≤2%) was observed with UGT1A1, UGT1A3, UGT1A4, UGT1A7, UGT1A8 and UGT1A10, when compared to UGT1A9 activity. UGT1A4 showed the highest rate of FLAVO-5-G formation, followed by UGT1A1 (19%) and UGT1A9 (12%). Flavopiridol glucuronidation activity was not observed using isoforms UGT1A5, UGT1A6, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28.

Human liver microsomes were capable of forming both flavopiridol glucuronides. The activities of FLAVO-7-G and FLAVO-5-G in human liver microsomes were 1997 ± 105 (98.5%) and 30 ± 4 pmol/min/mg (1.5%), respectively.

Kinetic Analyses

Kinetic characterization was performed for comparison of the glucuronidating activities of human liver microsomes and UGT1A9, which showed the highest reactivity for flavopiridol of all sixteen UGT isoenzymes tested. Apparent Michaelis-Menten kinetic parameters for the formation of flavopiridol glucuronides are shown in Table II. As illustrated in Fig. 3, the apparent different Y-intercepts (equal to $1/V_{max}$) and common X-intercept (equal to -1/apparent K_m) of the two plots suggest a slightly higher affinity for UGT1A9 (apparent $K_m = 37 \pm 6$ μM, $r^2 = 0.96$) than for human liver microsomes (apparent $K_m = 125 \pm 18$ μM, $r^2 = 0.99$). V_{max} values for UGT1A9 and human liver microsomes were 200 pmol/min/mg protein and 56 pmol/min/mg protein, respectively. Catalytic efficiencies (V_{max}/K_m) of 1.5 μL/min/mg and 1.6 μL/min/mg were observed for UGT1A9 and human liver microsomes, respectively.

Inter-Individual Variability of FLAVO-7-G Formation in Human Liver Microsomes

Inter-individual variation in the formation of FLAVO-7-G was evaluated using 62 human liver microsomes. The FLAVO-7-G/flavone peak height ratio varied twelvefold,

Table I. Formation Rates of FLAVO-7-G and FLAVO-5-G by Human UGT Enzymes

UGT enzymes	Absolute formation rate (pmol/min/mg)		Relative level of expressed UGT protein	Relative formation rate (pmol/min/mg)	
	FLAVO-7-G	FLAVO-5-G		FLAVO-7-G	FLAVO-5-G
UGT1A1	113 ± 13	9.0 ± 1.5	0.78	145 ± 16 (2%)	11.6 ± 1.9 (19%)
UGT1A3	20 ± 1		0.84	24 ± 1 (0.3%)	
UGT1A4	27 ± 1	15.0 ± 5.7	0.25	110 ± 3 (1.5%)	59.9 ± 22.7 (100%)
UGT1A7	16 ± 4		1.00	16 ± 4 (0.2%)	
UGT1A8	210 ± 12		1.34	156 ± 9 (2%)	
UGT1A9	1523 ± 92	1.5 ± 5.7	0.21	7253 ± 437 (100%)	7.1 ± 3.4 (12%)
UGT1A10	4 ± 1		0.05	87 ± 10 (1.2%)	

Note: Formation rates were determined by LC/MS using microsomal preparations from HEK293 cells. To better assess the relative contribution of individual UGT enzymes, glucuronidating activity towards flavopiridol was normalized by the level of expressed UGT protein in recombinant UGT-HEK293 cells as determined by Western blot analysis. The expression of UGT1A7 was arbitrarily designated as the basal UGT1A expression level. Data were expressed as the mean ± SD of two experiments performed in duplicate.

ranging from 0.11–1.27. The mean value (±S.D.) and coefficient of variation were 0.53 ± 0.26 and 49%, respectively. The frequency distribution was apparently normal (data not shown).

Correlation Studies with Propofol

The correlation between the glucuronidation of propofol and flavopiridol was determined in a subset of human liver samples. Propofol glucuronidating activity (propofol-G/*p*-fluorophenol peak height ratio) as assessed in 30 human liver microsomes, varied threefold, ranging from 0.20–0.66 (mean ± SD = 0.50 ± 0.12, coefficient of variation = 24%). A low (but significant) correlation was found between the *in vitro* formation of FLAVO-7-G and propofol-G ($r = 0.36$, $p = 0.048$) (Fig. 4).

Glucuronidation of Propofol by cDNA Expressed Isoforms of UGT

To determine if the correlation observed between propofol and flavopiridol glucuronidation rates in human liver microsomes was due to reactivity of additional UGT isoenzymes for propofol, propofol glucuronidation rates were assayed using nine isoforms: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B7, and UGT2B15. The enzymes UGT1A9, UGT1A7, UGT2B15 and UGT2B7 had propofol glucuronidating activity (propofol-G/*p*-fluorophenol peak height ratio) of 0.41 ± 0.00, 0.08 ± 0.03, 0.07 ± 0.00 and 0.05 ± 0.01, respectively. The isoforms UGT1A1, UGTA3, UGT1A4, UGT1A6 and UGT1A10 did not catalyze the formation of propofol-G.

Table II. Apparent Kinetic Parameters for the Glucuronidation of Flavopiridol by Human Liver Microsomes (HLM) and Human UGT1A9

UGT source	Apparent K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m (μl/min/mg)
HLM	125 ± 18	200 ± 22	1.6
UGT1A9	37 ± 6	56 ± 4	1.5

Note: The values of apparent K_m and V_{max} for the formation of flavopiridol glucuronides were determined using microsomal preparations from human livers and HEK293 cells. Formation of flavopiridol glucuronides was measured by TLC using radiolabeled UDPGA as previously described (14,15). Values were expressed as the mean ± SD of a single experiment performed in triplicate.

DISCUSSION

The main product (98.5%) of the glucuronidation reaction of flavopiridol in human liver microsomes is FLAVO-7-G. This metabolite has also been detected in the plasma from renal cancer patients, with levels ranging from 296–567 nM (5). This is consistent with evidence demonstrating that the

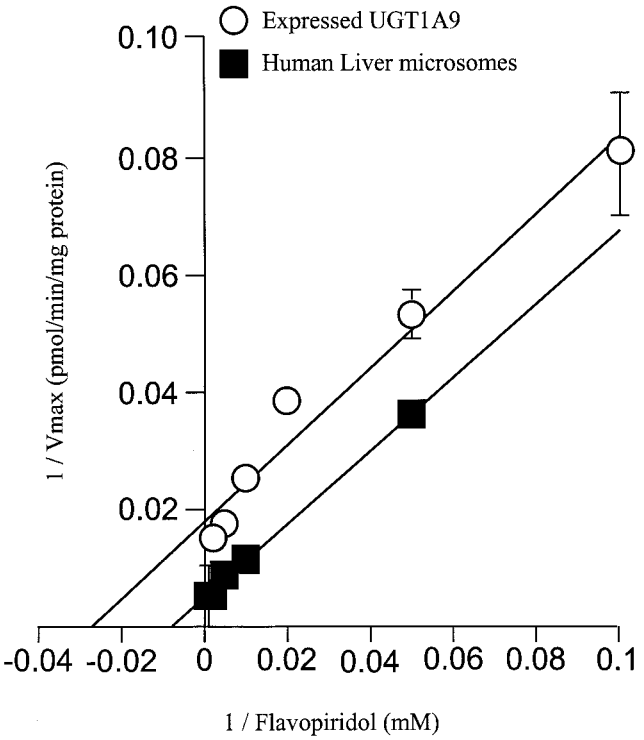

Fig. 3. Lineweaver-Burk plot of flavopiridol activities catalyzed by a pool of human liver microsomes and UGT1A9 membranes from HEK293 cells. Formation rates were determined using an assay with labeled co-substrate UDPGA and TLC. Values represent the mean ±SD of an experiment performed in triplicate. Correlation coefficients (r^2) of 0.99 and 0.96 were determined for human liver microsomes and UGT1A9 microsomes, respectively.

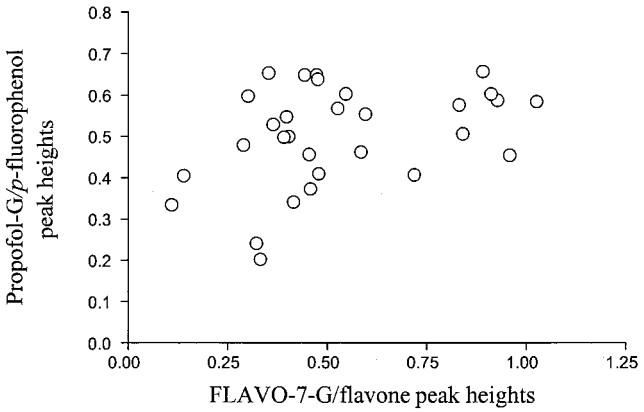

Fig. 4. Correlation analysis between the formation rates of FLAVO-7-G and propofol-G in 30 human liver microsomes.

7-hydroxyl position is the main conjugation site for the flavonoid acacetin, which is, like flavopiridol, a 5-7-dihydroxy compound (21).

The high variability (coefficient of variation = 49%) that we observed in the *in vitro* formation of FLAVO-7-G is in agreement with data from a clinical trial that showed high inter-patient differences (up to 99%) in the capacity to glucuronidate flavopiridol (5). Our data suggests that the variability in flavopiridol pharmacokinetics could be derived from differences in flavopiridol glucuronidation in the liver.

Inter-individual variation in drug response is often caused by genetic differences in drug-metabolizing enzymes (18,19). Identification of the enzyme(s) involved in the glucuronidation of flavopiridol and demonstration of the specificity of the reaction is of clinical relevance since enzyme variants might alter the susceptibility of patients to flavopiridol toxicity. A previous study reported that UGT1A9 is the main enzyme involved in the glucuronidation of flavopiridol after screening nine UGTs for their ability to glucuronidate flavopiridol (11). However, that study did not rule out the possible role in the glucuronidation of flavopiridol of seven UGTs, five of which are expressed in the liver (UGT2B4, UGT2B10, UGT2B11, UGT2B17 and UGT2B28) (9,10,20). After screening all existing UGTs for flavopiridol activity, we are able to clearly demonstrate the unique involvement of UGT1A9 in the glucuronidation of flavopiridol in humans, a conjugation reaction occurring mainly in the liver (4). Similar catalytic efficiencies found for the glucuronidation reaction with UGT1A9 and human liver microsomes provided additional evidence that UGT1A9 is the main enzyme involved in flavopiridol glucuronidation in the human liver. In contrast to the study of Hagenauer *et al.* (11), we found that (1) UGT1A1 was not significantly involved in the *in vitro* formation of FLAVO-7-G (2%), and (2) UGT1A4 is the main enzyme catalyzing the formation of FLAVO-5-G. This might be due to the fact that when determining the catalytic activity of the UGT enzymes for flavopiridol, we normalized the absolute formation rates by the level of expressed UGT proteins. In this way we were able to compare relative formation rates rather than absolute values.

Because propofol has been proposed as the probe substrate for UGT1A9 activity (17) and competitive inhibition of FLAVO-7-G formation by liver microsomes has been observed with propofol (11), we correlated the *in vitro* formation of FLAVO-7-G and propofol-G by human liver microsomes. We found a modest correlation between flavopiridol and propofol glucuronidation, which was explained by the activity of at least one additional hepatic UGT (UGT2B15) in the glucuronidation of propofol in addition to UGT1A9. Our data demonstrates that although propofol competitively inhibits the glucuronidation of flavopiridol (11), the glucuronidating activity of propofol does not reflect specifically the activity of UGT1A9 and thus should not be used to determine the phenotype of flavopiridol glucuronidation.

In summary, our data suggest that the variability in flavopiridol pharmacokinetics could be derived from differences in flavopiridol glucuronidation in the liver, and demonstrate the major role of UGT1A9 in the hepatic glucuronidation of flavopiridol. The variability observed in the *in vitro* formation of FLAVO-7-G could be indicative of the presence of a genetic polymorphism in UGT1A9, as suggested by the bimodality in metabolic ratios (ratio of concentrations of FLAVO-7-G/flavopiridol) observed in a flavopiridol trial (5). It has been shown that the extent of systemic flavopiridol glucuronidation is inversely related to the incidence of diarrhea (5). If a polymorphism in the *UGT1A9* gene is discovered and it could explain the unpredictable pharmacokinetics found for flavopiridol, pharmacogenetic screening prior to administration of flavopiridol might help to identify individuals predisposed to drug toxicity.

ACKNOWLEDGMENTS

This Pharmacogenetics of Anticancer Agents Group (pharmacogenetics.org) study was supported in part by grant GM61393 from the National Institutes of Health, Bethesda, MD. The primary data will be deposited into PharmGKB (www.pharmgkb.org), supported by grant (U01GM61374). This work was supported in part by the Canadian Institutes of Health Research (CIHR) and the Fonds de la Recherche en Santé du Québec (FRSQ). C.G. is a holder of a new investigator award from the Canadian Institutes of Health Research. Kim Journault is a recipient of the 'Fonds de l'enseignement et de la recherche' (FER) of the Faculty of Pharmacy, Laval University. We thank Drs. Thomas R. Tephly and Mitchell Green (University of Iowa) for providing the UGT2B7-HEK293 cell line.

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