UDP-glucuronosyltransferase 1A1 is the Principal Enzyme Responsible for Etoposide Glucuronidation in Human Liver and Intestinal Microsomes: Structural Characterization of Phenolic and Alcoholic Glucuronides of Etoposide and Estimation of Enzyme Kinetics

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Running title:

Etoposide glucuronidation is principally catalyzed by human UGT1A1

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Abbreviations used in this paper:

Etoposide, [4’-demethylepipodophyllotoxin-9-(4,6-O-(R)-ethyldiene-β-D-glucopyranoside)]; EG, etoposide glucuronide; EPG, etoposide phenolic glucuronide; EAG, etoposide alcoholic glucuronide; AZT, 3’-azido-3’-deoxythymidine; UGT, UDP-glucuronosyltransferase; UDPGA, uridine 5’-diphosphoglucuronic acid; D-SL, D-saccharic acid 1,4-lactone; DMSO, dimethyl sulfoxide; ACN,
acetonitrile; HLM, human liver microsome; HIM, human intestinal microsome; HPLC, high-performance liquid chromatography; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; TIC, total ion chromatogram; SIM, selective ion monitoring; IS, internal standard; \( t_R \), retention time.
ABSTRACT

Etoposide, an important anticancer agent, undergoes glucuronidation both in vitro and in vivo. In this study, three isomeric glucuronides of etoposide, including one phenolic (EPG) and two alcoholic glucuronides (EAG1 and EAG2), were biosynthesized in vitro with human liver microsomes (HLMs), and identified by liquid chromatography-electrospray ionization-mass spectrometry and confirmed by β-glucuronidase cleavage. In vitro UDP-glucuronosyltransferase (UGT) reaction screening with 12 recombinant human UGTs demonstrated that etoposide glucuronidation is mainly catalyzed by UGT1A1. Although UGT1A8 and 1A3 also catalyzed the glucuronidation of etoposide, their activities were about 10 and 1% of UGT1A1. Enzyme kinetic study indicated that the predominant form of etoposide glucuronide in HLMs and human intestinal microsomes (HIMs) was EPG, whereas EAG1 and EAG2 were the minor metabolites, with approximately 8-10% glucuronidation rate of EPG. For the formation of EPG, the $V_{\text{max}}$ of HLMs (110 pmol/min/mg protein) was very similar to that of recombinant UGT1A1 (124 pmol/min/mg protein), whereas the $V_{\text{max}}$ of HIMs (54.4 pmol/min/mg protein) was 2-fold lower than those of HIMs and UGT1A1. The $K_m$ values of HLMs (530 μM) and HIMs (608 μM) were 2-fold higher than that of UGT1A1 (285 μM). The $V_{\text{max}}/K_m$ values for the formation of EPG were 0.21 and 0.09 μl/min/mg protein for HLMs and HIMs, respectively. The data indicated that UGT1A1 is principally responsible for the formation of etoposide glucuronides, mainly in the form of phenolic glucuronide, suggesting that etoposide can be used as a highly selective probe substrate for human UGT1A1 in vitro.
Introduction

Glucuronidation represents a major conjugative reaction catalyzed by UDP-glucuronosyltransferases (UGTs). To date, 18 human UGT isoforms, including UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2A2, 2A4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28, have been identified (Mackenzie, et al., 1997; Finel, et al., 2005). Most of UGT enzymes are expressed in the liver, whereas UGT1A7, 1A8 and 1A10 are reported to be exclusively expressed in extrahepatic tissues (Radominska-Pandya, et al., 1999; Tukey and Strassburg, 2000; King, et al., 2000; Ritter, 2000). Human UGTs generally exhibit distinct but overlapping substrate specificities, and most substrates are glucuronidated by multiple UGT isoforms (Radominska-Pandya, et al., 1999; Tukey and Strassburg, 2000; King, et al., 2000; Ritter, 2000; Fisher, et al., 2001). Identification of human UGTs responsible for the glucuronidation of existing and novel drugs provides critical information about potential drug-drug interactions (Kiang, et al., 2005). Unlike cytochrome P450-mediated biotransformation, UGT reaction phenotyping has attracted less attention. Reasons for this include the limited availability of isoform-selective UGT substrates or antibodies and the relatively low incidence of drug-drug interactions involving UGT-catalyzed reactions in vivo (Williams, et al., 2004; Miners, et al., 2006). Several compounds, including bilirubin (Bosma, et al., 1994), β-estradiol (Senafi et al., 1994) and 17α-ethinylestradiol (Ebner et al., 1993), have been used as in vitro probe substrates for UGT1A1. However, bilirubin is a highly light-sensitive compound and forms multiple glucuronidation products, resulting in difficulties in separation and simultaneous determination (Miners, et al., 2004; Zhang, et al., 2005). Glucuronidations of β-estradiol and 17α-ethinylestradiol involve in
contributions of multiple UGT isoforms besides UGT1A1. Accumulating evidence indicates that UGT1A3, 1A4, 1A8, 1A10, 2B7 and 2B15 also metabolize β-estradiol glucuronidation (King, et al., 2000; Soars, et al., 2004), whereas UGT1A4, 1A8 and 1A9 may also catalyze the glucuronidation of 17α-ethinylestradiol (Cheng, et al., 1999; King, et al., 2000; Tukey and Strassburg, 2000). The lack of convenient and specific probe substrates for UGT1A1, which appears to have an important role in drug metabolism and drug-drug interactions, remains a problem (Miners, et al., 2004).

Etoposide [4’-demethylepipodophyllotoxin-9-(4,6-O-(R)-ethylidene-β-D-glucopyranoside)] (Fig. 1), a commonly used anticancer agent derived from epipodophyllotoxin, is mainly excreted as hydroxyl acid derivatives and glucuronides in humans after oral administration (Fleming, et al., 1989). Etoposide glucuronides account for the disposition of 15 to 35% of administered etoposide (D’lncalci, et al., 1986; Hande, et al., 1988b). Although several possible glucuronidation products of etoposide were found in humans and animals both in vitro and in vivo (Colombo, et al., 1985; D’lncalci, et al., 1986; Hande, et al., 1988a, 1988b), the structures of etoposide glucuronides and related enzyme responsibilities and kinetics involving human UGTs were not systematically or clearly identified. It has been recently reported that UGT1A1 is the specific catalyzing enzyme for the alcoholic glucuronidation of etoposide (Watanabe, et al., 2003), but the glucuronidation pathways of etoposide were not completely investigated with all known UGTs and multiple glucuronidation products were not observed. According to the chemical structure of etoposide, there are three hydroxyl groups (one phenolic and two alcoholic hydroxyls) that can potentially undergo glucuronidation (Fig. 1). To completely understand the glucuronidation pathways of etoposide,
we first biosynthesized the phenolic and alcoholic glucuronides of etoposide with human liver microsomes, and preliminarily characterized their structures by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and β-glucuronidase hydrolysis. A sensitive LC-ESI-MS assay for the simultaneous determination of etoposide glucuronides was developed using the biosynthesized standards. The isoforms responsible for the glucuronidation of etoposide were investigated using 12 commercially available recombinant human UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17), and human liver and intestinal microsomes. In vitro enzyme kinetics of etoposide glucuronidation in UGT1A1, 1A8, and human liver and intestinal microsomes were also estimated.

**Materials and Methods**

**Chemicals.** Etoposide [4’-demethylpipodophyllotoxin-9-(4,6-O-(R)-ethylidene-β-D-glucopyranoside)], 3’-azido-3’-deoxythymidine (AZT; internal standard for the quantification of etoposide and etoposide glucuronides), bilirubin, β-estradiol, 17α-ethinylestradiol, uridine 5’-diphosphoglucuronic acid (UDPGA), magnesium chloride, D-saccharic acid 1,4-lactone (D-SL; specific β-glucuronidase inhibitor), alamethicin, Tris-HCl (pH 7.4) and β-glucuronidase (type B-10 from bovine liver) were purchased from Sigma-Aldrich (St. Louis, MO). Etoposide glucuronides (EGs), including phenolic (EPG) and alcoholic glucuronides (EAG1 and EAG2), were biosynthesized using human liver microsomes, and separated and purified by a preparative HPLC method. Recombinant human UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17, BD Supersomes™ enzymes) were purchased from BD
Gentest (Woburn, MA). Pooled human liver microsomes (HLMs) were obtained from In Vitro Technologies (Baltimore, MD). Pooled human intestinal microsomes (HIMs) were purchased from XenoTech (Lenexa, Kansas). The protein contents of HLMs, HIMs and recombinant human UGTs were used as described in the data sheets provided by the manufacturers. Enzyme activities of recombinant human UGTs, HLMs and HIMs were confirmed by HPLC-UV assays as described by the manufacturers with modifications, using 7-hydroxy-4-trifluoromethylcoumarin as a substrate for 11 UGT isoforms (except UGT1A4), HLMs and HIMs, and trifluoperazine as a substrate for UGT1A4. Acetonitrile (ACN; HPLC grade) was obtained from Mallinckrodt (Phillipsburg, NJ). All other chemicals and reagents were of analytical grade.

**Glucuronidation of Etoposide.** In vitro glucuronidation of etoposide was conducted in an incubation volume of 200 µl. Incubation conditions were initially optimized using HLMs for linear product formation with respect to substrate concentrations (0-2000 µM), microsomal protein concentrations (0.25, 0.5, and 1 mg/ml), incubation time (15, 30, and 60 min) and activation of microsomes by alamethicin (10, 25, 50 and 100 µg/mg protein). The stock solution of etoposide was prepared in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the incubations was 1% (Preliminary experiments showed that 1% of DMSO in the final incubation has no influence on the glucuronidation of etoposide). Preliminary experiments indicated that the glucuronide formation of etoposide was linear up to 30 min of incubation time, 100 µg/mg protein of alamethicin and 0.5 mg/ml of microsomal protein.

Incubation mixtures containing 0.5 mg protein/ml of microsomes (recombinant human UGTs, HLMs and
HIMs), 0.1 M Tris-HCl buffer (pH 7.4) and 50 µg/mg protein of alamethicin in the final incubation (200 µl) were preincubated on ice for 10 min. After the addition of MgCl₂ (10 mM), D-SL (10 mM) and etoposide, the incubation mixture was preincubated at 37°C for 5 min. The reaction was initiated by the addition of UDPGA (2 mM) and incubated at 37°C in a shaking water bath for 30 min. The reaction was terminated by the addition of 800 µl of ice-cold ACN containing internal standard AZT (6.25 µg). After the removal of protein by centrifugation at 15,000g for 10 min at 4°C, the supernatants were transferred and dried with a stream of nitrogen at 45°C in a water bath. The residue was reconstituted in 125 µl of ACN/0.1% formic acid (20:80, v/v). The reconstituted samples were then centrifuged at 10,000g for 5 min at 4°C, and 20 µl of the final supernatants were introduced for LC-ESI-MS assay. Preliminary experiments indicated that the reconstituted samples containing etoposide, EGs and AZT in the reconstituting solvent were stable at least for 72 h at 4°C.

**Identification of Etoposide Glucuronides.** Structural identification of EGs was performed by LC-ESI-MS both in the positive and negative ionization modes with full scan detection using incubation samples of HLMs. HPLC separation was carried out using an Agilent HP 1050 LC system (Palo Alto, CA) with a guard column (Zorbax RX-C8, 12.5×4.6 mm i.d., 5 µm, Agilent, Palo Alto, CA) and an analytical column (Zorbax RX-C8, 150×12.1 mm i.d., 5 µm, Agilent, Palo Alto, CA). HPLC conditions: mobile phase, A-0.1% formic acid, B-ACN; gradient elution, 0-10 min, 10% B to 50% B, 10-12 min, 50% B, 12-14 min, 50% B to 10% B, 14-18 min, 10%B; column temperature, ambient; flow rate, 0.3 ml/min; injection volume, 20 µl; run time, 18 min. Typical retention times (t_R) of AZT, EPG, EAG1, EAG2 and...
etoposide under the experimental conditions used were 6.0, 8.0, 9.0, 9.5 and 10.5 min (Fig. 2), respectively.

MS analysis was performed by a PE Sciex API 100 liquid chromatography/mass spectrometry system (PE Sciex, Toronto, ON, Canada) with a TurboIonspray interface. MS parameters in the positive ESI ionization mode: ionspray voltage, 4500 V; ionspray temperature, 450°C; orifice voltage, 25 V; focusing ring voltage, 250 V; nebulizer gas, 10 l/min; curtain gas, 8 l/min; dwell time, 1 ms; pause time, 5 ms; scan time, 9 s; scan mode, full scan at the range of 100 to 1200 m/z. MS parameters in the negative ESI ionization mode: ionspray voltage, -3300 V; ionspray temperature, 450°C; orifice voltage, -50 V; focusing ring voltage, -250 V; nebulizer gas, 10 l/min; curtain gas, 8 l/min; dwell time, 1 ms; pause time, 5 ms; scan time, 9 s; scan mode, full scan at the range of 100 to 1200 m/z.

**Biosynthesis of Etoposide Glucuronides.** Enzymatic biosynthesis of EGs was conducted using HLMs. Briefly, 1 mM of etoposide was incubated with HLMs (2 mg protein/ml), 0.1 M Tris-HCl (pH 7.4), MgCl₂ (10 mM), Brij 35 (0.5 mg/mg protein), D-SL (10 mM) and UDPGA (2 mM) in 2×10 ml of final incubations for 7 h at 37°C. The stock solution of etoposide (0.5 M) was prepared in DMSO/MeOH (50:50, v/v). The concentration of organic solvent in the final incubation was 0.2%. The reaction was terminated by the addition of 2×30 ml of ice-cold ACN. After the removal of protein by centrifugation at 15,000g for 15 min at 4°C, the supernatants were pooled and dried with a stream of nitrogen at 45°C. The residue was reconstituted in 3 ml of ACN/1% acetic acid (20:80, v/v) and stored at -20°C until use.

Separation of EGs was performed by a preparative HPLC method using an Econosil C18 column.
(250×10 mm i.d., 10 µm, Alltech, Deerfield, IL) on a Shimadzu LC-6A HPLC system with UV detection. HPLC conditions used were as follows: mobile phase, ACN/1% acetic acid (23:77, v/v); elution mode, isocratic; flow rate, 4 ml/min; detection, 254 nm; injection volume, 800 µl; run time, 20 min. The retention times of EPG, EAG1 and EAG2 were 7, 10 and 13 min, respectively. After collection of the fractions of EGs (0.5-min intervals), the individual fractions corresponding to EPG, EAG1 and EAG2 confirmed by LC-ESI-MS were pooled and lyophilized. The lyophilized powders were dissolved in ACN/H₂O (50/50, v/v), and used as the standards for quantification. The purities of synthesized EGs estimated by both LC-ESI-MS and HPLC-UV (254 nm) assays were at least 97%.

The concentrations of synthesized EGs were estimated by LC-ESI-MS after β-glucuronidase cleavage. Briefly, aliquots of the diluted EGs standards were incubated with β-glucuronidase (10,000 U/ml in the final incubation) in 0.1 M NaAc buffer (pH 5) at 37ºC for 24 h. Preliminary experiments indicated that, under the enzymatic hydrolysis conditions tested, all forms of EGs could be completely cleaved to the parent compound, etoposide. The generated etoposide was quantified by LC-ESI-MS using etoposide calibration curve, and then the individual concentrations of EGs standards were estimated according to the molecular weight ratios of EGs to etoposide.

**Quantification of Etoposide Glucuronides.** Quantification of EGs was carried out by LC-ESI-MS in the negative ionization mode with selective ion monitoring (SIM) detection. HPLC conditions were the same as described in the identification of etoposide glucuronides. MS parameters used for quantification were as follows: ionspray voltage, -3300 V; ionspray temperature, 450 ºC; orifice voltage, -30 V; focusing ring
voltage, -200 V; nebulizer gas, 10 l/ml; curtain gas, 8 l/ml; dwell time, 300 ms; pause time, 5 ms; scan time, 1 s; scan mode, SIM with [M-H] for EGs (m/z 763), etoposide (m/z 587) and AZT (m/z 266), respectively.

Calibration curves were set up using the biosynthesized and purified standards of EGs. Standard solutions containing EPG, EAG1 and EAG2 were spiked into the incubation media, and then treated as described in the glucuronidation of etoposide. Concentrations of EGs in the samples were estimated with 1/x^2 weighted least-squares regression equations derived from the peak area ratios of EGs to that of AZT.

**UGT Reaction Screening of Etoposide.** UGT reaction screening of etoposide was conducted with HLMs, HIMs, 12 commercially available recombinant human UGTs and insect cell control (negative control) microsomes using a constant amount of microsomal protein (0.5 mg/ml) and a fixed concentration of etoposide (250 µM). Incubation conditions were the same as described in the glucuronidation of etoposide. Incubations without UDPGA or without substrate were also carried out. The incubation samples were treated as described in the glucuronidation of etoposide, and then analyzed by LC-ESI-MS.

**Enzyme Kinetics for the Glucuronidation of Etoposide.** Apparent enzyme kinetic parameters (K_m and V_max) for in vitro glucuronidation of etoposide were measured with recombinant human UGT1A1, 1A8, HLMs and HIMs, using various concentrations of etoposide (0-2000 µM) at fixed concentrations of microsomal proteins (0.5 mg/ml), MgCl_2 (10 mM), alamethicin (50 µg/mg protein), D-SL (10 mM) and UDPGA (2 mM) in a 200 µl of final incubation. Incubation conditions and sample preparation were similar to that of glucuronidation of etoposide described previously. The concentrations of EGs were
quantified by LC-ESI-MS. Apparent $K_m$ and $V_{max}$ values were estimated by fitting the duplicate experimental data to a Michaelis-Menten equation: $V = (V_{max} \times S) / (K_m + S)$, or a substrate inhibition equation (Houston and Kenworthy, 2000): $V = (V_{max} \times S) / (K_m + S + S^2/K_{si})$, where $K_m$ is the Michaelis-Menten constant, $V_{max}$ is the maximum velocity, $S$ is the substrate concentration, and $K_{si}$ is the substrate inhibition constant, by a non-linear least-squares regression using SigmaPlot (SPSS Inc., Chicago, IL). The apparent kinetic parameters were reported as mean ± standard error (SE) from individual duplicates.

**Inhibitory Effects of Typical Probe Substrates on Etoposide UGT Activity in HLMs.** Bilirubin (Bosma, et al., 1994), β-estradiol (Senafi et al., 1994; Soars, et al., 2003) and 17α-ethinylestradiol (Ebner et al., 1993), UGT1A1 substrates commonly used, were selected to investigate their potential inhibitory effects on etoposide UGT1A1 activity. The stock solutions of all substrates tested were prepared in DMSO, and the final concentration of DMSO in the incubation was 1%. The formation rates of etoposide glucuronides in HLMs (0.5 mg protein/ml) at a fixed concentration of etoposide (250 µM) with and without inhibitor (0-2000 µM) were determined by LC-ESI-MS. Incubation conditions were the same as described previously in glucuronidation of etoposide, and the $IC_{50}$ values were estimated graphically.

**Results**

**Structural Characterization of Etoposide Glucuronides.** Three isomeric glucuronidation products of etoposide were found in the incubations with HLMs and UGT1A1 (Fig. 2), and identified by LC-ESI-MS both in the positive and negative ion modes. In the positive ion mode, etoposide and its glucuronides
exhibited relatively abundant and multiple fragments, including protonated podophyllotoxin ion (m/z 383, the loss of glucose or sugar moiety from etoposide) and its subfragmentation ion (m/z 229), and several adducts of ammonium, sodium and potassium (Fig. 3). The proposed in-source MS fragmentation pathway of etoposide in the positive ion mode is shown in Figure 4, which is consistent with published reports (Chen and Uckun, 2000; Pang, et al., 2001). In the negative ion mode, all etoposide glucuronides exhibited a strong and exclusive base peak corresponding to the deprotonated intact glucuronide molecule ion ([M-H], m/z 763), whereas etoposide showed the deprotonated podophyllotoxin ion (m/z 381, the loss of glucose or sugar moiety from etoposide) as the predominant peak (Fig. 5). The diagnostic fragmentations for the formation of etoposide glucuronides were based on the fragmentation ion at m/z 589 ([M+H-176]+; the neutral loss of glucuronide moiety, 176 Da) and several adducts of ammonium ([M+NH₄]+, m/z 782), sodium ([M+Na]+, m/z 787) and potassium ([M+K]+, m/z 803) in the positive ion mode, and the fragmentation ion at m/z 587 ([M-H-176]-; the neutral loss of glucuronide moiety, 176 Da), the deprotonated intact glucuronide molecule ion ([M-H], m/z 763) and the loss of H₂O from [M-H]- (m/z 745) in the negative ion mode, respectively. In addition, enzyme hydrolysis showed that all conjugation products of etoposide could be cleaved into the parent compound by β-glucuronidase, further confirming the formation of glucuronide conjugates. The distinguishing fragments for the phenolic (EPG) and alcoholic glucuronides (EAG1 and EAG2) of etoposide were elucidated by the presence (phenolic glucuronide) or absence (alcoholic glucuronide) of the subfragmentation ions of m/z 435 and 247 resulting from the loss of glucuronide moiety and then the loss of phenolic side chain (Fig. 3 and 4b) and
subsequently the loss of glucose or sugar moiety (Fig. 3 and 4f) in the positive ion mode, and the distinctive cleavage of glucuronic acid moiety (m/z 175 and 113 for EPG; m/z 193, 175 and 113 for EAG1 and EAG2) in the negative ion mode (Figs. 5 and 6), respectively. Since the C-O bond to the aliphatic chain of alcoholic glucuronides is more easily cleaved than the C-O bond to the aromatic ring of phenol-linked glucuronides (Fenselau and Johnson, 1980; Niemeijer, et al., 1991), the MS spectra of EAG1 and EAG2 were very similar and did exhibit the deprotonated glucuronic acid ion (m/z 193), whereas EPG did not. These different in-source MS fragmentation pathways of glucuronic acid moieties in etoposide phenolic and alcoholic glucuronides in the negative ion mode (Fig. 6) were in agreement with previous findings about phenolic and alcoholic glucuronides (Niemeijer, et al., 1991; Gu, et al., 1999; Levsen, et al., 2005). Additional evidence for the identification of the positional isomers of phenolic and alcoholic glucuronides is their different chromatographic retentions on reversed-phase HPLC, as phenolic glucuronides (e.g., EPG $t_R = 8.0$ min) are generally more hydrophilic, and consequently have earlier elution than those of alcoholic glucuronides (e.g., EAG1 and EAG2, $t_R = 9.0$ and 9.5 min, respectively). The similar examples include estradiol 3- and 17-glucuronides (Alkharfy and Frye, 2002), ethinylestradiol 3- and 17-glucuronides (Ebner, et al., 1993), and the phenolic and alcoholic glucuronides of denopamine (Kaji and Kume, 2005) and labetalol (Niemeijer, et al., 1991). However, the conjugation sites of the two alcoholic glucuronides of etoposide could not be individually identified by MS methods, and this need to be further elucidated. In addition, in this study, the formation of di- or tri-glucuronides of etoposide was not observed under the experimental conditions.
Quantification of Etoposide Glucuronides. Experiments indicated that all etoposide glucuronides had higher MS responses in the negative ionization mode than that of the positive ionization mode. Quantification of EGs was then performed with SIM detection in the negative ionization mode. The limit of detection was 0.5 ng/ml for all forms of EGs with injection volume of 20 µl. The quantitative linear ranges were 2 to 1500 ng/ml for EPG, and 1 to 1000 ng/ml for EAG1 and EAG2. The intra-day precisions (relative standard deviation, n = 4) were 1.5-5.6%, 1.0-6.4% and 1.1-7.0% for EPG, EAG1 and EAG2, respectively. The inter-day precisions (relative standard deviation, n = 4) were 1.5-6.8%, 1.2-4.0% for and 2.9-13% for EPG, EAG1 and EAG2, respectively. Experimentally, the most important MS parameters for the quantification of intact glucuronides were the cone voltages, including orifice and focusing ring voltages, in this study. Increasing cone voltages significantly enhanced MS intensities of molecular ions of etoposide and EGs (data not shown). However, at high cone voltages (> -40 and -300 V of orifice and focusing ring voltages, respectively), the MS intensity of EGs significantly decreased, because some of the intact glucuronides underwent in-source dissociation. Increasing the ionspray voltage (from -2000 to -3300 V) resulted in slightly increase of MS responses of etoposide and EGs, but the influence was much less than those of cone voltages. Changing the ionspray temperature (from 250 to 450 ºC) showed no or little effect on the determination sensitivity. No significant in-source thermal degradation of EGs was observed at high ionspray temperature (450 ºC) when the optimal cone voltages were used in this study. It is noted that the MS responses of EPG, EAG1 and EAG2 were apparently different from each other at the same concentrations (EAG2 > EPG > EAG1), and also quite different from their parent compound,
etoposide. The cone voltage-dependent sensitivities for the quantification of intact etoposide glucuronides by LC-ESI-MS are consistent with the analysis of other glucuronides in biological samples (Yan, et al., 2003). Collectively, the effects of cone voltages on the determination of sensitivity by LC-ESI-MS are chemical structure and molecular weight dependent, as relatively low cone voltages are beneficial to the quantification of intact glucuronides (e.g., EPG, EAG1 and EAG2), while high cone voltages are helpful for the determination of the parent or unconjugated compounds (e.g., etoposide).

**UGT Reaction Screening of Etoposide.** Incubation of etoposide (250 µM) with 12 commercially available recombinant human UGTs, insect cell control (negative control) microsomes, HLMs and HIMs demonstrated that three UGT isoforms (UGT1A1, 1A8 and 1A3), HLMs and HIMs catalyzed the glucuronidation of etoposide in vitro (Fig. 7). No glucuronidation of etoposide was observed with other nine UGT isoforms tested and the insect cell control microsomes. Three etoposide glucuronides were found in vitro, with the predominant form of etoposide glucuronide being the phenolic glucuronide (EPG). Alcoholic glucuronides (EAG1 and EAG2) were the minor metabolites, with approximately 8-10% glucuronidation rate of EPG formation (Figs. 2 and 7). Among the three UGT isoforms that catalyzed the glucuronidation of etoposide, UGT1A1 had the highest glucuronide formation rates (62, 7.1 and 6.9 pmol/min/mg protein for EPG, EAG1 and EAG2, respectively). The activities of UGT1A8 (6.1, 0.37 and 3.7 pmol/min/mg protein for EPG, EAG1 and EAG2, respectively) and UGT1A3 (0.60, 0.64 and 0.05 pmol/min/mg protein for EPG, EAG1 and EAG2, respectively) were much lower than those of UGT1A1.

**Enzyme Kinetic Parameters.** Apparent enzyme kinetic parameters were estimated using various
concentrations of etoposide (0-2000 μM) with recombinant human UGT1A1, 1A8, HLMs and HIMs. The kinetics of UGT1A1, HLMs and HIMs fitted to the Michaelis-Menten equation at 5-2000 μM etoposide (Fig. 8 and Table 1). For the formation of EPG catalyzed by UGT1A1, the $V_{\text{max}}$, $K_m$ and $V_{\text{max}}/K_m$ values were 124 pmol/min/mg protein, 285 μM and 0.44 μl/min/mg protein, respectively. In comparison with UGT1A1, the $V_{\text{max}}$ of EPG with HLMs (110 pmol/min/mg protein) was very similar, but its $K_m$ (530 μM) was about 2-fold higher, resulting in a 2-fold lower value of $V_{\text{max}}/K_m$ (0.21 μl/min/mg protein). The formation rate of EPG in the incubation containing HIMs (54.4 pmol/min/mg protein) was about 50% of HLMs and UGT1A1. The enzyme activities toward the formation of alcoholic glucuronides of etoposide (EAG1 and EAG2) by UGT1A1, HLMs and HIMs were much lower, with 4- to 13-fold lower $V_{\text{max}}$ values than that of EPG (Figs. 2 and 7, and Table 1). The $K_m$ of EAG1 with UGT1A1 (230 μM) was slightly lower than that of EPG (285 μM), whereas the $K_m$ values of EAG1 with HLMs (330 μM) and HIMs (324 μM) were very similar. The $K_m$ values of EAG2 with UGT1A1 (753 μM), HLMs (1010 μM) and HIMs (922 μM) were approximately 2- to 3-fold higher than that of EPG and EAG1.

The kinetics of etoposide glucuronidation in UGT1A8 at 5 to 500 μM etoposide fitted to the typical Michaelis-Menten kinetics, with the $V_{\text{max}}$ and $K_m$ values of 13.4 pmol/min/mg protein and 65 μM for EPG and 5.28 pmol/min/mg protein and 83 μM for EAG2 (Fig. 9 and Table 2), respectively. However, when the etoposide concentration exceeded 500 μM, the formation rates of etoposide glucuronides (EPG and EAG2) gradually decreased with the increase of etoposide concentration, exhibiting a substrate inhibition kinetics (Fig. 9). When the apparent enzyme kinetic parameters were estimated by fitting to the substrate
inhibition kinetics at 5 to 2000 µM etoposide, the $V_{\text{max}}$, $K_m$ and $K_i$ (substrate inhibition constant) values were 19.1 pmol/min/mg protein, 113 and 1004 µM for EPG, and 6.89 pmol/min/mg protein, 125 and 1421 µM for EAG2 (Table 2), respectively. The enzyme kinetics for the formation of EAG1 catalyzed by UGT1A8, and the kinetics of UGT1A3 for etoposide glucuronidation were not estimated due to their very low activities.

**Inhibition of Etoposide Glucuronidation by UGT1A1 Probe Substrates.** Inhibitory effects of three commonly used UGT1A1 probe substrates, bilirubin, β-estradiol and 17α-ethinylestradiol, on etoposide UGT activity in HLMs are shown in Fig. 10. The estimated $IC_{50}$ values are summarized in Table 3. The substrates tested exhibited different inhibitory influence on the formation of individual etoposide glucuronides in vitro. Bilirubin, the putatively specific substrate of UGT1A1, strongly inhibited the formation of EPG ($IC_{50} = 75$ µM) and EAG1 ($IC_{50} = 54$ µM), and significantly inhibited the formation of EAG2 ($IC_{50} = 140$ µM). 17α-Ethinylestradiol strongly inhibited the formation of EAG2 ($IC_{50} = 42$ µM), but showed very similar and moderate inhibition on EPG ($IC_{50} = 200$ µM) and EAG1 ($IC_{50} = 210$ µM). β-Estradiol also exhibited strong inhibition on EPG ($IC_{50} = 68$ µM) and EAG2 ($IC_{50} = 86$ µM). Interestingly, the inhibitory effect of β-estradiol on the formation of EAG1 was not so significant in comparison with other two etoposide glucuronide isomers, even at very high concentration of β-estradiol (~80% at 2000 µM). Collectively, these inhibitory results suggested that etoposide is the selective substrate of UGT1A1.
Human UGTs generally exhibit distinct but overlapping substrate specificities, and most substrates are glucuronidated by more than one isoform (Radomska-Pandya, et al., 1999; Tukey and Strassburg, 2000; King, et al., 2000; Ritter, 2000; Fisher, et al., 2001). Due to the lack of specific antibodies for human UGT isoforms, it is important to alternatively explore some selective substrates for the evaluation of the responsibility of individual UGT isoforms. Currently, several compounds, including bilirubin (Bosma, et al., 1994), β-estradiol (Senafi et al., 1994) and 17α-ethynylestradiol (Ebner et al., 1993), have been used as the probe substrates for UGT1A1 in vitro. Bilirubin is putatively considered as the most specific substrate for UGT1A1 (Bosma, et al., 1994; Burchell, et al., 1995; King, et al., 2000). However, simultaneous and accurate determination of bilirubin and its multiple glucuronidation isomers (including one di-glucuronide, and 2 cis/trans and 2 positional isomers of mono-glucuronides) is difficult due to the light sensitivity of bilirubin, and the instability or interconversion of mono- and di-glucuronide of bilirubin in vitro and in vivo (Jansen, et al., 1977; Chowdhury, et al., 1981; Gordon, et al., 1984; Miners, et al., 2004; Zhang, et al., 2005). In addition, it has been reported that bilirubin may also inhibit UGT1A4 (Ghosal, et al., 2004).

UGT1A1 predominantly catalyzes the phenolic glucuronidation of β-estradiol (Senafi et al., 1994; Soars, et al., 2003) at the position 3 with atypical kinetics, whereas UGT2B7 mainly catalyzes the alcoholic glucuronidation of β-estradiol at the position 17 (Gall, et al., 1999). However, UGT1A8, 1A10 and 1A3 have been also reported to catalyze the glucuronidation of β-estradiol-3 with significant activities (Soars, et al., 2004), while UGT1A3, 1A4, 1A8, 1A10 and 2B7 may also be involved in the glucuronidation of
β-estradiol-17 (Soars, et al., 2004). Although 17α-ethinylestradiol glucuronidation is primarily catalyzed by UGT1A1 (Ebner et al., 1993), other UGT isoforms (e.g., UGT1A4, 1A8 and 1A10) may also metabolize the glucuronidation of 17α-ethinylestradiol at lower but significant activities (Cheng, et al., 1999; King, et al., 2000; Tukey and Strassburg, 2000). This overlapping substrate specificity for “probes” of UGT1A1 complicates the interpretation of in vitro data, particularly in human intestinal tissues where relatively high levels of UGT 1A8 and 1A10 are expressed (Cheng, et al., 1999; Radomska-Pandya, et al., 1999; Tukey and Strassburg, 2000). Since multiple UGT enzymes catalyze the glucuronidation of β-estradiol and 17α-ethinylestradiol, caution should be taken when these two compounds are chosen as the probe substrates of UGT1A1.

It has been recently reported that UGT1A1 only catalyzes the alcoholic glucuronidation of etoposide (Watanabe, et al., 2003), but the glucuronidation pathways of etoposide were still unclear, considering its potential multiple glucuronidation products (Fig. 1). Additionally, since etoposide glucuronide standards were not utilized, true Michaelis-Menten parameters were not determined. In this study, we reported that three positional isomeric glucuronides of etoposide (Fig. 2), including one phenolic (EPG) and two alcoholic glucuronides (EAG1 and EAG2), were formed in the incubations containing recombinant human UGTs (UGT1A1, 1A3 and 1A8), HLMs and HIMs in vitro (Fig. 7). The main glucuronidation product of etoposide was the phenolic glucuronide (EPG), whereas the two alcoholic glucuronides (EAG1 and EAG2) were the minor metabolites. The formation of multiple etoposide glucuronides in vitro found in this study is consistent with the previous in vivo results observed in rats and rabbits after administration.
of etoposide (Colombo, et al., 1985; Hande, et al., 1988a), although the structures of etoposide glucuronides were not completely characterized in the previous publications. In this report, the structures of etoposide glucuronides were preliminarily identified by LC-ESI-MS both in the positive and negative ionization modes, and confirmed by β-glucuronidase cleavage, although the conjugation sites of the two alcoholic glucuronides of etoposide need to be further distinguished. Our data also indicated that, at the same concentrations, etoposide and its three glucuronides had different MS responses (EAG2 > EPG > EAG1 > etoposide), when measured in the negative ionization mode. Therefore, accurate quantification of etoposide glucuronides using the biosynthesized standards is necessary. UGT reaction screening (Fig. 7) and enzyme kinetic study (Figs. 8 and 9, and Tables 1 and 2) indicated that three UGT isoforms, including UGT1A1, 1A3 and 1A8, were responsible for the formation of etoposide glucuronides in vitro. UGT1A1 is the principal enzyme catalyzing etoposide glucuronidation, whereas the enzyme activities of UGT1A3 and 1A8 were only 1 and 10% of UGT1A1, respectively, although the individual contribution of the related UGT isoforms needs to be further confirmed and quantitatively compared considering the expressed levels among the different isoforms. The Eadie-Hofstee plots for HLMs and HIMs (Fig. 8D, E, and F) showed the involvement of a single UGT isoform, UGT1A1, in the etoposide glucuronidation reactions, suggesting the contributions of UGT1A8 or 1A3 might be minor. In addition, inhibitory study also indicated that etoposide is the selective substrate of bilirubin glucuronidating isoform, UGT1A1 (Fig. 10 and Table 3).

In conclusion, we demonstrated that three isomeric glucuronides of etoposide were formed in vitro,
including one phenolic and two alcoholic glucuronides. UGT1A1 is the principal enzyme responsible for the formation of etoposide glucuronides in vitro, mainly in the form of phenolic glucuronide. Considering the catalyzing activities of UGT1A8 and 1A3 for the glucuronidation of etoposide are much lower than that of UGT1A1, and UGT1A8 is mainly expressed in extrahepatic tissues, etoposide could be used as an alternative or better selective substrate for human UGT1A1 than other non-specific probes in hepatic systems.
References


Kiang TKL, Ensom MHH and Chang TKH (2005) UDP-glucuronosyltransferases and clinical drug-drug


Footnotes

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Legends for figures

**FIG. 1.** Chemical structure and proposed glucuronidation pathways of etoposide.

**FIG. 2.** Representative LC-ESI-MS chromatograms (TIC, total ion chromatogram) for the formation of etoposide glucuronides in pooled human liver microsomes (HLMs) and recombinant human UGT1A1. A, HLMs without UDPGA; B, HLMs; C, recombinant UGT1A1; peak 1, etoposide phenolic glucuronide (EPG); peaks 2 and 3, etoposide alcoholic glucuronides (EAG1 and EAG2). Typical retention times of AZT, EPG, EAG1, EAG2 and etoposide under the experimental conditions were 6.0, 8.0, 9.0, 9.5 and 10.5 min, respectively.

**FIG. 3.** MS spectra of etoposide and its glucuronides in the positive ion mode. LC-ESI-MS analysis was conducted as described under *Materials and Methods*. A-etoposide, B- etoposide phenolic glucuronide (EPG), C and D- etoposide alcoholic glucuronides (EAG1 and EAG2).

**FIG. 4.** Proposed MS fragmentation pathway of etoposide in the positive ion mode.

**FIG. 5.** MS spectra of etoposide and its glucuronides in the negative ion mode. LC-ESI-MS analysis was conducted as described under *Materials and Methods*. A-etoposide, B-etoposide phenolic glucuronide (EPG), C and D-etoposide alcoholic glucuronides (EAG1 and EAG2).
FIG. 6. Proposed MS fragmentation pathways of glucuronic acid moieties in etoposide phenolic and alcoholic glucuronides in the negative ion mode.

FIG. 7. UGT reaction screening of glucuronidation of etoposide with 12 recombinant human UGTs, insect control microsomes, pooled human liver microsomes (HLMs) and pooled human intestinal microsomes (HIMs). The concentrations of etoposide in the final incubations were 250 µM. The formation rates of etoposide glucuronides were determined as described under Materials and Methods. Data were expressed as pmol/min/mg protein with the means of individual duplicates.

FIG. 8. Enzyme kinetics for the glucuronidation of etoposide with pooled human liver microsomes (HLMs), pooled human intestinal microsomes (HIMs) and recombinant human UGT1A1. The formation rates of etoposide glucuronides were determined as described under Materials and Methods. Data were expressed as pmol/min/mg protein with the means of individual duplicates. A, B and C; the Michaelis-Menten plots for EPG, EAG1 and EAG2, respectively. D, E and F; the Eadie-Hofsteet plots for EPG, EAG1 and EAG2, respectively.

FIG. 9. Enzyme kinetics for the glucuronidation of etoposide with recombinant human UGT1A8. The formation rates of etoposide glucuronides (EPG and EAG2) were determined as described under...
Materials and Methods. The kinetics for the formation of EAG1 catalyzed by UGT1A8 was not measured due to very low activity. Data were expressed as pmol/min/mg protein with the means of individual duplicates. A and B; the Michaelis-Menten plots for EPG and EAG2, respectively. The dotted lines represent the fitting curves to the Michaelis-Menten equation at 5 to 500 µM etoposide (closed circles). The solid lines represent the fitting curves to the substrate inhibition equation at 5 to 2000 µM etoposide (closed and open circles). C and D; the Eadie-Hofstee plots for EPG and EAG2, respectively.

FIG. 10. Inhibitory effects of typical probe substrates on etoposide UGT activity in pooled human liver microsomes (HLMs). Etoposide UGT activities at 250 µM etoposide were determined as described under Materials and Methods. Bilirubin (0-750 µM), β-estradiol (0-2000 µM) and 17α-ethinylestradiol (0-2000 µM) were used as inhibitors. Data (means of individual duplicates) were expressed as peak area ratios relative to internal standard (AZT) in comparison with the controls without inhibitors. A, bilirubin; B, β-estradiol; C, 17α-ethinylestradiol.
**TABLE 1**

*Kinetic parameters for the formation of etoposide glucuronides with recombinant human UGT1A1 and pooled human liver and intestinal microsomes*

<table>
<thead>
<tr>
<th>Etoposide Glucuronide</th>
<th>Enzyme Source</th>
<th>$V_{\text{max}}$ (pmol/min/mg protein)</th>
<th>$K_{m}$ (µM)</th>
<th>$V_{\text{max}}/K_{m}$ (µl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPG</td>
<td>UGT1A1</td>
<td>124 ± 4</td>
<td>285 ± 34</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>HLMs</td>
<td>110 ± 12</td>
<td>530 ± 57</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>HIMs</td>
<td>54.4 ± 5</td>
<td>608 ± 123</td>
<td>0.089</td>
</tr>
<tr>
<td>EAG1</td>
<td>UGT1A1</td>
<td>12.5 ± 0.5</td>
<td>230 ± 31</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>HLMs</td>
<td>8.79 ± 0.3</td>
<td>330 ± 38</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>HIMs</td>
<td>4.04 ± 0.2</td>
<td>324 ± 36</td>
<td>0.012</td>
</tr>
<tr>
<td>EAG2</td>
<td>UGT1A1</td>
<td>26.3 ± 1.4</td>
<td>753 ± 90</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>HLMs</td>
<td>16.4 ± 0.7</td>
<td>1010 ± 90</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>HIMs</td>
<td>16.5 ± 1.7</td>
<td>922 ± 190</td>
<td>0.018</td>
</tr>
</tbody>
</table>

HLMs, pooled human liver microsomes; HIMs, pooled human intestinal microsomes.

Enzyme kinetic parameters were estimated by fitting to the Michaelis-Menten kinetics at 5 to 2000 µM etoposide. Data were measured as described under *Materials and Methods* and expressed as mean ± standard error ($V_{\text{max}}$ and $K_{m}$) or means ($V_{\text{max}}/K_{m}$) of individual duplicates.
TABLE 2

Kinetic parameters for the formation of etoposide glucuronides with recombinant human UGT1A8

<table>
<thead>
<tr>
<th>Etoposide Glucuronide</th>
<th>Michaelis-Menten Kinetics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Substrate Inhibition Kinetics&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>EPG</td>
<td>13.4 ± 0.6</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>EAG2</td>
<td>5.28 ± 0.2</td>
<td>83 ± 11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enzyme kinetic parameters were estimated by fitting to the Michaelis-Menten kinetics at 5 to 500 µM etoposide.

<sup>b</sup> Enzyme kinetic parameters were estimated by fitting to the substrate inhibition kinetics at 5 to 2000 µM etoposide.

Data were measured as described under Materials and Methods and expressed as mean ± standard error ($V_{\text{max}}$, $K_m$ and $K_{\text{si}}$) or means ($V_{\text{max}}/K_m$) of individual duplicates. $V_{\text{max}}$, pmol/min/mg protein. $K_m$ and $K_{\text{si}}$, µM. $V_{\text{max}}/K_m$, µl/min/mg protein. $K_{\text{si}}$, substrate inhibition constant. The kinetics for the formation of EAG1 catalyzed by UGT1A8 was not measured due to very low activity.
TABLE 3
Estimated IC₅₀ (µM) of typical inhibitors on etoposide (250 µM) UGT activity in human liver microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>EPG</th>
<th>EAG1</th>
<th>EAG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin</td>
<td>75</td>
<td>54</td>
<td>140</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>68</td>
<td>&gt;2000</td>
<td>86</td>
</tr>
<tr>
<td>17α-Ethinylestradiol</td>
<td>200</td>
<td>210</td>
<td>42</td>
</tr>
</tbody>
</table>

Data were measured as described under Materials and Methods and expressed as the means of individual duplicates.
**Fig. 1**

Glucuronide moiety (glu)

\[
\text{H}_3\text{C} - \text{O} - \text{O} - \text{OH} - \text{O} - \text{OH} - \text{O} - \text{OH}
\]

H\text{Alcoholic glucuronide (EAG)}

\[
\text{R}_1 \quad \text{R}_2 \quad \text{R}_3
\]

<table>
<thead>
<tr>
<th>Phenolic glucuronide (EPG)</th>
<th>glu</th>
<th>H</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic glucuronide (EAG)</td>
<td>H</td>
<td>glu</td>
<td>H</td>
</tr>
<tr>
<td>Alcoholic glucuronide (EAG)</td>
<td>H</td>
<td>H</td>
<td>glu</td>
</tr>
</tbody>
</table>

UGTs

UDPGA

UDP
Fig. 2

A

AZT (IS)

Etoposide

Response (cps)

Retention time (min)

B

AZT (IS)

Etoposide

C

AZT (IS)

Etoposide
Fig. 4

\[ [\text{M+NH}_4]^+, m/z \ 606 \]
\[ [\text{M+Na}]^+, m/z \ 611 \]
\[ [\text{M+K}]^+, m/z \ 627 \]

\[ [\text{M+H}]^+, m/z \ 589, C_{29}H_{33}O_{13} \]

\[ \text{m/z} \ 383, C_{21}H_{19}O_{7} \]
\[ \text{m/z} \ 299, C_{17}H_{15}O_{5} \]

\[ \text{m/z} \ 229, C_{13}H_{9}O_{4} \]
\[ \text{m/z} \ 247, C_{13}H_{11}O_{5} \]
\[ \text{m/z} \ 185, C_{12}H_{9}O_{2} \]
Fig. 6

Phenolic glucuronide

Alcoholic glucuronide

m/z 175

m/z 113

m/z 193

m/z 175

m/z 113
Fig. 7
Fig. 8

(A) V (pmol/min/mg protein) vs. S (µM) for HLM, HIM, and UGT1A1.

(B) V (pmol/min/mg protein) vs. S (µM) for HLM, HIM, and UGT1A1.

(C) V (pmol/min/mg protein) vs. S (µM) for HLM, HIM, and UGT1A1.

(D) V/S (µl/min/mg protein) vs. S (µM) for HLM, HIM, and UGT1A1.

(E) V/S (µl/min/mg protein) vs. S (µM) for HLM, HIM, and UGT1A1.

(F) V/S (µl/min/mg protein) vs. S (µM) for HLM, HIM, and UGT1A1.
Fig. 9

The graphs illustrate the relationship between reaction velocity (V) and substrate concentration (S) at different V/S values, measured in pmol/min/mg protein and µl/min/mg protein, respectively.