KINETICS AND REGULATION OF CYTOCHROME P450-MEDIATED ETOPOSIDE METABOLISM

Xiaoliang Zhuo,1 Naifyu Zheng,2 Carolyn A. Felix, and Ian A. Blair

Center for Cancer Pharmacology, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania (X.Z., N.Z., I.A.B.); and Division of Oncology, the Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania (C.A.F.)

Received March 4, 2004; accepted June 15, 2004

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Etoposide is a DNA topoisomerase II inhibitor widely used in the treatment of a variety of malignancies that is also associated with therapy-related leukemia. The cytochrome P450 (P450)-derived catechol and quinone metabolites of etoposide may be important in the damage to the MLL (mixed lineage leukemia) gene and other genes resulting in leukemia-associated chromosomal translocations. Kinetic analysis of catechol formation by recombinant P450s was determined using liquid chromatography/selected reaction monitoring/mass spectrometry. CYP3A4 was found to play a major role in etoposide metabolism (Km = 77.7 ± 27.8 μM; Vmax = 314 ± 84 pmol of catechol/min/nmol of P450). However, CYP3A5 (Km = 13.9 ± 3.1 μM; Vmax = 19.4 ± 0.4 pmol of catechol/min/nmol of P450) may be involved in etoposide metabolism at therapeutic concentrations of free drug. Other P450s do not appear to be involved in etoposide catechol formation. Real-time polymerase chain reaction and Western blot analysis revealed significantly increased CYP3A4 mRNA and protein levels in hepatocytes treated with 10 μM rifampicin compared with untreated cells, but only modest effects of rifampicin on CYP3A5 induction. Etoposide (40, 5, 1, and 0.25 μM) caused a slight increase in CYP3A4 mRNA in three of five batches of hepatocytes but did not result in proportionately increased CYP3A4 protein levels. At high concentrations, etoposide induced only a modest increase in CYP3A5 mRNA and protein levels in four of five batches of hepatocytes. Alternatively, coadministration of other drugs with etoposide may account for the increase in etoposide catechol formation during therapy with etoposide.

Etoposide is a DNA topoisomerase II inhibitor that decreases the religation rate in the DNA topoisomerase II cleavage-religation equilibrium (Fortune and Osheroff, 2000). As a semisynthetic analog of the natural product podophyllotoxins, found in mandrake plants, etoposide has been widely used and highly efficacious in anticancer treatment since the 1980s (Hande, 1998). Like many other anticancer drugs, etoposide is administered as a single agent or, more often, as a component of combined regimens. However, treatment with etoposide has been linked to leukemias with balanced chromosomal translocations as a consequence of treatment. Many of the translocations disrupt the MLL (mixed lineage leukemia) gene at chromosome band 11q23. Chromosomal breakage resulting from drug-induced stabilization of the DNA topoisomerase II cleavage complex has been proposed as the DNA damage mechanism leading to these translocations (Felix, 1998; Lovett et al., 2001a,b; Whitmarsh et al., 2003).

This work was supported in part by National Institutes of Health Grant R01CA77683-04 as well as the Pediatric Pharmacology Research Unit of the Children’s Hospital of Philadelphia National Institutes of Health Grant U01 5-U01-HD-37259.

1 Current address: Department of Discovery Biotransformation, Bristol-Myers Squibb Company, 5 Research Parkway, Wallingford, CT 06492.

2 Current address: Department of Clinical Discovery Analytical Sciences, Bristol-Myers Squibb Company, 1 Squibb Drive, New Brunswick, NJ 08903.

O-Demethylation by cytochromes P450 (P450s) represents the major pathway of etoposide metabolism, producing 3-hydroxyetoposide (etoposide catechol), which undergoes sequential one-electron oxidations to form a semiquinone and then a quinone moiety (Fig. 1) (van Maanen et al., 1988; Mans et al., 1990). Previously, we have shown that more DNA topoisomerase II cleavage is induced by etoposide catechol and etoposide quinone at certain cleavage sites in the MLL gene than by the parent drug (Felix, 1998; Lovett et al., 2001a,b). The DNA topoisomerase II-dependent and site-selective nature of the damage by the drug metabolites proximal to translocation breakpoints may support a crucial role of etoposide metabolism in leukemogenesis. Among human P450s, CYP3A4 has been demonstrated to be the principal isoform involved in etoposide O-demethylation, whereas CYP3A5, CYP2E1, and CYP1A2 have been suggested to play minor roles (Relling et al., 1992, 1994; Kawashiro et al., 1998). However, enzyme kinetic data for CYP3A5-, CYP2E1-, and CYP1A2-mediated metabolism are lacking and the relative roles of these enzymes in vivo are unknown. Therefore, evaluating the relative importance of these P450s is critical for pharmacogenetic studies directed at understanding the link between etoposide metabolism and the risk of etoposide-induced leukemia.

Our previous pharmacokinetic studies demonstrated that protein-free and total (plasma bound + free) plasma etoposide catechol
concentrations increased significantly in pediatric patients undergoing conventional multiple-day bolus dosing (100 mg/m²/day) with etoposide on day 5 compared with day 1 (Pang et al., 2001; Zheng et al., 2004). Since CYP3A4, CYP2E1, and CYP1A2 are all induced by different categories of xenobiotics (Rendic and Di Carlo, 1997), this observation suggested that the enzymes responsible for etoposide catechol formation might be induced by the multiple-day chemotherapy. Schuetz et al. (2002) reported that in livers of transgenic mice, rifampicin and etoposide can increase luciferase gene expression driven by a 7.8-kb 5’-flanking region of CYP3A4, and that this up-regulation was mediated by the pregnane X receptor. The mechanism for up-regulation of CYP3A5 expression has not been studied extensively even though CYP3A5 and CYP3A4 have overlapping substrate specificity (Rendic and Di Carlo, 1997), and earlier studies were hampered because the 5’-flanking sequence was incorrectly characterized (Jounaidi et al., 1994). However, the correct sequence of the CYP3A5 5’-flanking region has now been established (Finta and Zaphiropoulos, 2000).

Here, kinetic analysis of etoposide catechol formation by recombinant P450s was conducted using a highly sensitive, specific method based on liquid chromatography-tandem mass spectrometry (LC/MS/MS). The ability of etoposide to up-regulate the expression of P450 enzymes involved in etoposide metabolism was determined. Studies were conducted by monitoring the levels of P450 proteins and their corresponding mRNAs in primary cultures of human hepatocytes.

Materials and Methods

Etoposide and Catechol Metabolite. Etoposide was obtained from Sigma-Aldrich (St. Louis, MO). Etoposide catechol was synthesized according to a method devised in our laboratory (Pang et al., 2001). Briefly, etoposide was dissolved in dioxane/water and demethylated with sodium metaperiodate in the dark at 4°C. The resulting quinone was extracted with dichloromethane and crystallized by adding ether. Etoposide quinone was then dissolved in dioxane/water under an atmosphere of nitrogen and reduced to the corresponding catechol with sodium borohydride. It was recrystallized with dichloromethane/ether and stored in the dark until used.

Kinetic Analysis of Etoposide Metabolism. Reaction mixtures (200 μl) contained 100 mM potassium phosphate buffer (pH 7.4), 2 mM ascorbic acid, and etoposide (Sigma-Aldrich; 10, 25, 50, 63, 83, 125, 250, or 500 μM for CYP3A4; 6.25, 12.5, 25, 50, 63, 83, 125, or 250 μM for CYP3A5; 2.5, 5, 10, 15, 20, 40, 60, or 120 μM for CYP2E1; and 120 μM for CYP1A2, CYP2B6, or CYP2A6; different etoposide concentrations were used due to the variability of enzyme activities). No cytochrome b₅ was coexpressed with P450s and reductase. The reaction systems also contained recombinant human P450s (BD Gentest, Woburn, MA): CYP3A4 (30 nM), CYP3A5 (30 nM), CYP2E1 (60 nM), CYP1A2 (60 nM), CYP2A6 (30 nM), or CYP2B6 (60 nM) with coexpressed human NADPH-cytochrome P450 reductase. After a 2-min preincubation, the reactions were triggered by adding an NADPH-generating system (3.3 mM MgCl₂, 3.3 mM glucose 6-phosphate, 1.3 mM NADP⁺, and 0.4 unit of glucose-6-phosphate dehydrogenase as final concentrations) according to the BD Gentest protocol, and carried out at 37°C for 15 min (CYP3A4), 30 min (CYP3A5 or CYP2E1), or 60 min (CYP1A2, CYP2A6, or CYP2B6), during which the etoposide catechol formation rate was within a linear range. The reactions were terminated by adding 800 μl of ice-cold acetonitrile, and then 200 ng of teniposide (Bristol-Myers Squibb Co., Stamford, CT) was added as an internal standard. After centrifugation at 12,000 rpm in a microcentrifuge for 10 min, 950-μl supernatants were removed to new Eppendorf tubes and dried using a nitrogen stream. The residues were dissolved in 200 μl of 65% H₂O/35% methanol containing 0.1% HCOOH and 5 mM HCOONH₄, and the solutions were sonicated for 3 min followed by injection of 50 μl into the
LC/MS/MS system. A seven-point calibration curve of etoposide catechol was prepared for quantitative purposes, and 200-μl reaction systems. Individual P450s were spiked with an equal amount of etoposide catechol (Pang et al., 2001) to final concentrations from 5 to 1000 pg/μl, and with 200 ng of teniposide. Calibration samples were extracted in exactly the same manner as experimental samples.

Quantitative Analysis of Etoposide Catechol using LC/MS/MS. Quantitation of etoposide catechol was performed on a Waters 2690 Alliance high-pressure liquid chromatography system (Waters, Milford, MA) equipped with a Finnigan TQ 7000 triple-quadrupole mass spectrometer (Thermo Electron, Franklin, MA) equipped with a Finnigan electrospray ionization source. Separation of etoposide, etoposide catechol, and teniposide was achieved on a YMC (Wilmington, NC) ODS-AQ analytical column (150 × 2.0 mm i.d., 3 μm) using mobile phase A (10% acetonitrile/90% H2O) and B (90% acetonitrile/10% H2O), both containing 5 mM HCOONa, and 0.1% HCOOH. The high-pressure liquid chromatography elution method and parameters applied for tandem mass spectrometry, including spray voltage, capillary temperature, and collision energy, were previously described (Pang et al., 2001).

The peak areas were calculated using Finnigan ICIS 8.3.0 software.

Primary Cultures of Human Hepatocytes and Treatment with P450 Inducing Agents. The use of commercially available anonymous primary human hepatocytes from unlinked human adult donors was exempt from Institutional Review Board review and approval. Primary cultures of human hepatocytes in six-well plates (collagen-coated) were purchased from Tissue Transformation Technologies (Edison, NJ), In Vitro Technologies, Inc. (Baltimore, MD), Cambrex Bio Science Baltimore, Inc. (Baltimore, MD), or BD Gentest. Information on the donors, including age, gender, race, weight, medical condition, and drug history, is listed in Table 1. Upon receipt, the hepatocytes were incubated at 37 °C in 5% CO2 and 95% humid atmosphere for 4 h, and the medium was then replaced with 3.5 ml of fresh hepatocytes maintenance medium (HMM) (Cambrex Bio Science Baltimore, Inc.) supplemented with 100 nM dexamethasone, 100 nM insulin, 50 μg/ml gentamicin, and 50 ng/ml amphoterin B. The purpose of the dexamethasone was to maintain the phenotype of constitutive P450 expression and regulation (Lecluyse, 2001). The control P450 induction agent rifampicin (Sigma-Aldrich) or etoposide was dissolved in DMSO and diluted in HMM to different concentrations. Due to the impact of cell-plating density on P450 induction, plating densities of 125,000 to 150,000 cells/cm2 were used as recommended (Lecluyse, 2001). The cell density was determined with a hemacytometer by trypsin blue exclusion. Hepatocytes were only used if they were in this range and they were no more than 5 days old when they were received from the suppliers. The plating density was ≥80% (≥1.5 × 106 cells/well). No significant cell loss during 48 h of incubation was detected under the light microscope in the presence or absence of inducing agents. Therefore, plating densities were 125,000 to 150,000 cells/cm2 during the incubations. Approximately 48 h after plating, the cells were treated with rifampicin (10 μM), etoposide, or 0.1% DMSO in HMM. The final concentration of DMSO in each inducer preparation was 0.1% (v/v). Since our previous pharmacokinetic studies indicated that the maximum concentrations of protein-free and total etoposide in plasma in pediatric patients treated with 100 mg/m2/day, daily for 5 days by intravenous bolus dosing were 1.4 μM and 40 μM, respectively (Pang et al., 2001), the final etoposide concentrations were 40, 5, and 0.25 μM. Medium containing inducers or DMSO was renewed daily and treatment was performed for 48 h.

Preparation of S9 Fraction. To detach the hepatocytes, the media was drained from the wells and 1 ml of phosphate-buffered saline containing 0.05% trypsin (0.25%) and 1 mM EDTA was added to each well. Hepatocytes were harvested by centrifugation at 2000 rpm for 3 min and resuspended in 350 μl of S9 fraction preparation buffer [100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 15 mM potassium, 15 mM EDTA, 1% (v/v) manganous protease inhibitor cocktail, and 0.5 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich)] (Liu et al., 2001). Cell disruption was performed using a Polytron tissue homogenizer (Brinkmann, Westbury, NY) and an ultrasonic (Misonix Incorporated, Farmingdale, NY) followed by centrifugation at 9,000 g for 10 min at 4 °C. Three hundred microliters of supernatants (postmitochondrial S9 fraction) were transferred into Eppendorf tubes and stored at −80 °C until used. The protein concentrations of S9 fractions were determined using the BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a protein standard via absorbance determination at 562 nm in a Beckman DU530 UV-visible spectrometer (Beckman Coulter, Fullerton, CA).

Reverse Transcriptase PCR. The HMM was drained from wells of six-well plates, and 1 ml of TRIzol Reagent (Invitrogen, Carlsbad, CA) was directly added to each well for cell lysis. Total RNA was isolated from hepatocytes according to the protocol of the manufacturer and suspended in 50 μl of diethylpyrocarbonate-treated H2O. The total RNA (1.5–2 μg) from each preparation was treated for 30 min at 37 °C, with 2 U of RNase-free DNase I (Roche Diagnostics, Indianapolis, IN) and 20 U of RNase inhibitor in 1× PCR buffer II (Applied Biosystems, Foster City, CA), followed by heat inactivation at 75 °C for 5 min. First-strand cDNA was synthesized by reverse transcribing at 42 °C for 60 min using reagents provided in the GeneAmp RNA PCR core kit and random hexamers according to the protocol of the manufacturer (Applied Biosystems). Primer Express software (version 2.0.0) (Applied Biosystems) was used to design CYP3A4- and CYP3A5-specific primers, sequences of which are listed in Table 2. Since the CYP3A4 and CYP3A5 cDNAs share 90% sequence identity, primers were selected from exon 1 and exon 3, where there is a 16% sequence variation.

Conventional PCR was conducted to examine primer specificity. Each 20-μl PCR mixture contained 1× PCR buffer II, 2 mM MgCl2, 0.5 mM deoxynucleoside-5′-triphosphates, 0.5 μl of cDNA, 0.5 mM primers, and 1 U of AmpliTaq DNA polymerase (Applied Biosystems). PCR conditions were as follows: 95 °C for 5 min followed by 30 cycles consisting of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. The final elongation was conducted at 72 °C for 7 min. PCR products were examined on 2% agarose gels and directly sequenced using automated methods.

Quantitative Real-Time PCR Analysis. cDNA was synthesized as above and PCR primers were added to 96-well optical reaction plates (Applied Biosystems). Quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems). The 40 thermal cycles included heat denaturation at 95 °C for 15 s, and combined annealing and extension at 60 °C for 60 s. The melting temperatures of the CYP3A4, CYP3A5, and GAPDH amplicons were 81.3, 82.5, and 80.4 °C, respectively. Each reaction was performed in triplicate. PCR primer concentrations were optimized at 200 nM to minimize nonspecific amplification and the formation of primer dimers on fluorescent absorbance. The seven-point relative standard curves for CYP3A4, CYP3A5 and GAPDH quantitation were generated using 1-, 2-, 4-, 8-, 16-, 32-, and 64-fold serial dilutions of the cDNA from the hepatocytes of donor HH11 that had been treated with 10 μM rifampicin.

Western Blot Analysis of P450 Levels. S9 fractions containing equal concentrations from the cDNAs from the hepatocytes of donor HH11 that had been treated with 10 μM rifampicin.

### TABLE 1

<table>
<thead>
<tr>
<th>Donor Identification</th>
<th>Sex</th>
<th>Age</th>
<th>Race</th>
<th>Weight</th>
<th>Medical History</th>
<th>Drug Exposure</th>
<th>Concentrations of Etoposide</th>
<th>P450 Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH5</td>
<td>Female</td>
<td>58</td>
<td>Caucasian</td>
<td>184</td>
<td>Hypertension, mini stroke</td>
<td>N/A</td>
<td>40</td>
<td>mRNA and protein</td>
</tr>
<tr>
<td>HH7</td>
<td>Female</td>
<td>49</td>
<td>Caucasian</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>40</td>
<td>mRNA and protein</td>
</tr>
<tr>
<td>HH8</td>
<td>Male</td>
<td>31</td>
<td>Caucasian</td>
<td>140</td>
<td>Asthma</td>
<td>Paxil, cercueil</td>
<td>5, 1, and 0.25</td>
<td>Protein</td>
</tr>
<tr>
<td>HH9</td>
<td>Male</td>
<td>53</td>
<td>Caucasian</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>5, 1, and 0.25</td>
<td>mRNA and protein</td>
</tr>
<tr>
<td>HH10</td>
<td>Male</td>
<td>47</td>
<td>Caucasian</td>
<td>95</td>
<td>Type II diabetes</td>
<td>N/A</td>
<td>5, 1, and 0.25</td>
<td>mRNA and protein</td>
</tr>
<tr>
<td>HH11</td>
<td>Female</td>
<td>49</td>
<td>Caucasian</td>
<td>68</td>
<td>N/A</td>
<td>Smoker, drinker</td>
<td>40, 5, 1, and 0.25</td>
<td>mRNA and protein</td>
</tr>
<tr>
<td>HH13</td>
<td>Male</td>
<td>43</td>
<td>Caucasian</td>
<td>43</td>
<td>N/A</td>
<td>Smoker, drinker</td>
<td>40, 5, 1, and 0.25</td>
<td>mRNA</td>
</tr>
</tbody>
</table>

N/A, not available.
CYP3A4 exhibited the highest catalytic efficiency (1.2 pmol of etoposide catechol/min/nmol of P450, respectively. CYP3A5 and CYP2E1 were 13.9 Vm/Vm, H9262 human CYP3A5 polyclonal antibody (1:500 dilution), and goat anti-rat CYP2E1 reference for CYP3A recognition. The antibodies (BD Gentest) included rabbit anti-human CYP2A6 were tested. At etoposide concentrations between 2.5 and 10 ng/ml. Etoposide, etoposide catechol, and teniposide were resolved by the reversed-phase high-pressure liquid chromatography within 10 min. The retention time and selected reaction monitoring transition used for analyzing each compound are indicated.

**Results**

**Kinetic Analysis of Etoposide Metabolism by Human P450s.** An LC/MS/MS method using multiple reaction monitoring was validated for the simultaneous determination of etoposide and etoposide catechol, with a lower limit of quantitation for etoposide catechol of 2.5 ng/ml. Etoposide, etoposide catechol, and teniposide were resolved by the reversed-phase high-pressure liquid chromatography within 10 min (Fig. 2) and selectively detected using structurally significant product ions (Fig. 3). Catalytic activities toward etoposide of recombinant human CYP3A4, CYP3A5, CYP2E1, CYP1A2, CYP2B6, and CYP2A6 were tested. At etoposide concentrations between 2.5 and 120 μM, only CYP3A4, CYP3A5, and CYP2E1 displayed activity, whereas the activities of CYP1A2, CYP2B6, and CYP2A6 were negligible or undetectable. Kinetic analyses using Lineweaver-Burk double-reciprocal plots (Fig. 4) showed that the K_m and V_max values for CYP3A4 were 77.7 ± 27.8 μM and 314 ± 84 pmol of etoposide catechol/min/mmol of P450, respectively (Table 3). The K_m values for CYP3A5 and CYP2E1 were 13.9 ± 3.1 μM and 26.0 ± 10.5 μM, respectively. However, the V_max values for CYP3A5 and CYP2E1 were significantly lower than that of CYP3A4 at 19.4 ± 0.4 and 3.4 ± 1.2 pmol of etoposide catechol/min/mmol of P450, respectively. CYP3A4 exhibited the highest catalytic efficiency (V_max/K_m) in etoposide catechol formation among the P450s that were tested (Table 3).

**Effect of Etoposide on P450 mRNA Levels in Human Hepatocytes.** In the analyses of P450 induction in primary cultures of hepatocytes, neither rifampicin nor etoposide treatment was associated with increased cell death or loss of cell adhesion observed using light microscopy. The generation of PCR products of expected sizes and verification of the relevant CYP transcript sequences in the conventional PCR products confirmed the specificity of the primers shown in Table 2 (data not shown). Upon treatment of hepatocytes from five of the donors (HH7, HH9, HH10, HH11, and HH13) with the known CYP3A4 inducer rifampicin at a concentration of 10 μM, the mean increase in CYP3A4 mRNA levels was 38.7-fold (Fig. 5) (range 7.9- to 79.9-fold). Treatment with 10 μM rifampicin also caused an increase in CYP3A5 mRNA, but to a much lesser extent (1.3-fold to 3.1-fold). Rifampicin caused statistically significant increases in CYP3A4 mRNA levels in human hepatocytes (p = 0.031) (Fig. 5), but not in CYP3A5 mRNA levels (p = 0.060) (Fig. 6). Compared with rifampicin, etoposide had a much weaker effect on CYP3A4 mRNA induction (Fig. 5). Treatment with etoposide was associated with a modest (≤50%) increase in CYP3A4 mRNA levels in hepatocytes from donors HH10, HH11, and HH13, whereas less than 50% increase was found in the hepatocytes from the other donors tested (HH7 and HH9). The greatest increase in CYP3A4 mRNA relative to the DMSO control was 235% observed in hepatocytes from donor HH11, treated with etoposide at 0.25 μM. The induction of CYP3A4 mRNA by etoposide at the different concentrations from 0.25 to 40 μM was not significantly different (p > 0.30), indicating lack of a dose-response effect. The effect of etoposide on CYP3A5 mRNA levels is shown in Fig. 6. Etoposide treatment increased CYP3A5 mRNA levels by 50% to 84% in hepatocytes isolated from donors HH9, HH10, and HH11, but no increase or less than 50% increase was observed in hepatocytes from donors HH7 and HH13. Overall, etoposide treatment did not cause significant CYP3A4 and amounts of protein (5–10 μg, depending on protein concentrations of S9 fractions), and recombinant P450 standards were separated on a 10% Tris-Glycine gel (Invitrogen) using an XCell SureLock Mini-Cell electrophoresis system (Invitrogen) and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Approximately 0.05 to 0.2 pmol of CYP3A4 or CYP3A5 with 5 μg of bovine serum albumin was loaded on the gel, mainly to serve as a reference for CYP3A recognition. The antibodies (BD Gentest) included rabbit anti-human CYP3A4 polyclonal antibody (1:500 dilution), rabbit anti-human CYP3A5 polyclonal antibody (1:500 dilution), and goat anti-rat CYP2E1 polyclonal antibody (1:1000 dilution). The blots were re-probed with mouse anti-human β-actin monoclonal antibody (Sigma-Aldrich). Immunoreactive bands were detected using enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ). Band intensities were quantified using ImageQuant v5.0 (Amersham Biosciences Inc.).

**Data Analysis.** In the analysis of in vitro kinetic constants of etoposide O-demethylation, reactions were repeated three times for each P450. The K_m and V_max values were calculated based on least-squared linear regression of [1/(etoposide concentration)] and [1/(etoposide catechol formation rate) plots, expressed as the mean ± S.D., and analyzed statistically by the paired Student’s t test. P450 mRNA levels in treated and untreated human hepatocytes were normalized to GAPDH levels, and P450 protein levels were normalized to β-actin levels. Fold induction was represented as a ratio of P450 mRNA or protein level in hepatocytes treated with either rifampicin or etoposide to that in hepatocytes treated with vehicle alone. Paired t tests were performed to assess whether rifampicin or etoposide causes significant increases in P450 mRNA and protein levels in human hepatocytes.

**TABLE 2**

Specific primers used for real-time PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>GenBank Accession Number</th>
<th>Sequence</th>
<th>Size of PCR Product (base pairs)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4E1F</td>
<td>13904851</td>
<td>5’ ATAGTTAAGGAACTATGAGCTGCCTCA 3’</td>
<td>200</td>
<td>Exon 1–3</td>
</tr>
<tr>
<td>3A4E3R</td>
<td></td>
<td>5’ TCAAGTTAAGGAACTATGAGCTGCCTCA 3’</td>
<td>201</td>
<td>Exon 1–3</td>
</tr>
<tr>
<td>3A5E1F</td>
<td>15147331</td>
<td>5’ TCCGTTAAGGAACTATGAGCTGCCTCA 3’</td>
<td>78</td>
<td>Exon 1–2</td>
</tr>
<tr>
<td>3A5E3R</td>
<td></td>
<td>5’ GACCTGTTAAGGAACTATGAGCTGCCTCA 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>BC004109</td>
<td>5’ GAGGTGTTAAGGAACTATGAGCTGCCTCA 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>BC004109</td>
<td>5’ CAGAGTTAAGGAACTATGAGCTGCCTCA 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bowen et al. (2000).
CYP3A5 mRNA increases \((p < 0.06)\) in human hepatocytes. The effect of etoposide treatment on CYP2E1 mRNA levels was not evaluated in this study.

**Effect of Etoposide on CYP3A4, CYP3A5, and CYP2E1 Protein Expression in Human Hepatocytes.** Western blot analysis was used to detect CYP3A4 protein in hepatocytes from donors HH5, HH7, HH8, HH9, HH10, and HH11, and CYP3A5 protein in hepatocytes from donors HH6, HH7, HH8, HH9, HH10, and HH11. The detection of CYP3A4 and CYP3A5 proteins in human hepatocytes from donors HH10 and HH11 is illustrated in Fig. 7. Although there were some nonspecific bands, the relevant immunoactive bands were recognized by comigration with the corresponding protein standards. Treatment with 10 \(\mu M\) rifampicin significantly elevated CYP3A4 protein levels \((p < 0.04)\) by a mean of 17-fold over the DMSO control (range 1.8- to 36-fold). Rifampicin-mediated induction of CYP3A4 protein was correlated with rifampicin-mediated mRNA induction, except in the hepatocytes from donor HH9. In contrast, treatment with 10 \(\mu M\) rifampicin elevated CYP3A5 protein levels only by a mean of 2.5-fold over the DMSO control, which was not statistically significant \((p < 0.08)\).

Treatment with etoposide had very little effect \((<37\%)\) on CYP3A4 protein levels except in hepatocytes from subject HH11, in which treatment with etoposide at 5 \(\mu M\) was associated with a 65\% increase (Fig. 5B). Treatment with etoposide at 40 \(\mu M\) and 5 \(\mu M\) was associated with 50\% to 90\% increases in CYP3A5 protein expression.

**TABLE 3**

<table>
<thead>
<tr>
<th>P450s</th>
<th>(K_m)</th>
<th>(V_{max})</th>
<th>(V_{max}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>77.7 ± 27.8(^a)</td>
<td>314 ± 84(^b)</td>
<td>4.0</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>13.9 ± 3.1(^c)</td>
<td>19.4 ± 0.4(^d)</td>
<td>1.4</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>26.0 ± 10.5</td>
<td>3.4 ± 1.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Statistically different from \(K_m\) of CYP3A5 and CYP2E1 (\(p < 0.033\)).

\(^{b}\) Statistically different from \(V_{max}\) of CYP3A5 and CYP2E1 (\(p < 0.04\)).

\(^{c}\) No different from \(K_m\) of CYP2E1 (\(P = 0.128\)).

\(^{d}\) Statistically different from \(V_{max}\) of CYP2E1 (\(p < 0.017\)).
in hepatocytes from donors HH7, HH8, HH9, and HH11 (Fig. 6). Although statistical analysis showed significant increase in CYP3A4 and CYP3A5 protein levels in hepatocytes treated with 1 and 5 μM etoposide, respectively, the average increase was less than 54%. Etoposide treatment was not associated with any increase in CYP2E1 protein greater than 50% over the DMSO control (data not shown).

**Discussion**

Catechol formation represents the major P450-mediated metabolism pathway of etoposide. Etoposide catechol is rapidly oxidized to the corresponding quinone. However, the presence of 2 μM ascorbic acid in our in vitro reaction systems converted all etoposide quinone back to the catechol metabolite. Therefore, in the present study, it was possible to quantify the combined amount of etoposide catechol and etoposide quinone using a highly sensitive and selective LC/MS/MS method. CYP3A4 was shown to have the highest \( V_{\text{max}} \) and catalytic efficiency when compared with CYP3A5 and CYP2E1. The catalytic activity of CYP1A2, CYP2B6, and CYP2A6 was below the detection limit of the assay. The \( K_m \) value of CYP3A4 was not significantly different from that determined previously (Kawashiro et al., 1998); however, there was a statistically significant difference between the \( V_{\text{max}} \) values (\( p = 0.005 \), likely due to the different analytical method used in the earlier study (Kawashiro et al., 1998). Numerous studies have been conducted over the last 30 years to assess the pharmacokinetic and pharmacodynamic effects of etoposide (Creaven, 1982; Sinkule, 1984; McLeod and Evans, 1993; Hande, 1998). Previously we (Pang et al., 2001; Zheng et al., 2004) and others (Creaven, 1982; Sinkule, 1984; McLeod and Evans, 1993; Hande, 1998) have demonstrated that more than 95% of etoposide is bound to plasma protein in vivo, like many other anticancer agents. Although...
the total etoposide (free and protein bound) concentration maximum ($C_{\text{max}}$) reached 40 μM in patient plasma, the protein-free etoposide (unbound) $C_{\text{max}}$ was only 1.4 to 2.0 μM. The $K_{\text{u}}$ value of CYP3A4 for etoposide catechol formation was significantly lower than that of CYP3A4 (Table 3). This suggests that CYP3A5 is a significant contributor to catechol formation at the free etoposide concentrations found in subjects undergoing therapy. CYP2E1 will play only a minor role in etoposide metabolism due to its low catalytic efficiency. It is known that cytochrome $b_5$ can influence the activity of CYP3A enzymes (Yamaori et al., 2003). Therefore, omission of cytochrome $b_5$ in the reconstituted systems may have influenced the relative catalytic activities of CYP3A4 and CYP3A5 in etoposide catechol formation.

The previously reported correlation between etoposide O-demethylation and testosterone 6β-hydroxylation in human liver microsomes does not exclude the possible involvement of CYP3A5 because CYP3A5 is also involved in testosterone 6β-hydroxylation (Kawashiro et al., 1998). Kuehl et al. (2001) demonstrated that CYP3A5 accounts for 50% of CYP3A protein content in 30% of Caucasian and 50% of African-American livers. However, a recent study suggested that CYP3A5 content determined by Kuehl et al. (2001) might have been overestimated for Caucasians (Westlind et al., 2003). On balance, it appears that the presence or absence of hepatic CYP3A5 could be an important determinant of overall etoposide metabolism.

A protective effect of the single nucleotide A to G polymorphism in the nifedipine-sensitive response element of the CYP3A4 promoter and association of the wild-type CYP3A4 promoter genotype with an increased risk of treatment-related leukemia have been reported by our group (Felix et al., 1998). This CYP3A4 promoter-genotype association was validated in a study of Israeli adults with treatment-related leukemia with MLL translocations (Ben-Yehuda et al., 2002). The same association was not observed in a population of pediatric patients who developed treatment-related acute myeloid leukemia following primary acute lymphoblastic leukemia therapy with etoposide or teniposide-containing regimens (Blanco et al., 2002). In addition, no association with CYP3A5 genotype with the risk of treatment-related leukemia was observed in the latter population. A recent study of children with primary acute lymphoblastic leukemia has found that concomitant treatment with prednisone can also increase the clearance of etoposide with an increase in formation of the catechol metabolites (Kishi et al., 2004). Furthermore, the same study has suggested that the wild-type (AA) genotype at the nifedipine-sensitive response element of the CYP3A4 promoter is associated with lower etoposide clearance in Caucasians at week 54 in the absence of prednisone, and with a higher catechol area under the curve in both Caucasians and African Americans at day 29 while on prednisone. Among African Americans on prednisone on day 29, the CYP3A4 GG genotype was associated with lower etoposide clearance. The CYP3A5 AA genotype was associated with higher etoposide catechol area under the curve at day 29 while on prednisone and with lower etoposide clearance at week 54 off prednisone (Kishi et al., 2004).

Human hepatocytes retain all of the transcription factors to support both constitutive and regulatory expression of xenobiotic-metabolizing enzymes in a species-specific fashion. Despite their limited availability, primary culture of human hepatocytes provides a valuable in vitro cell model for studying P450 regulation. Induction studies using 10 μM rifampicin in primary cultures of human hepatocytes showed increases of CYP3A4 mRNA and protein levels similar to those reported previously (Chang et al., 1997; Raucy, 2003). The impact of etoposide on P450 regulation was much lower and was only observed in hepatocytes from some individuals. Etoposide can induce apoptosis in tumor cell lines (Brown and Wouters, 1999; Grzanka et al., 2003) at the higher concentrations that were used in the induction studies (40 μM). However, etoposide at 40 μM was not cytotoxic to the human hepatocytes when evaluated by light microscopy. There are no published data describing etoposide effects on cytotoxicity or apoptosis in human hepatocytes. A study conducted on rat hepatocytes showed that no apoptosis (as measured by caspase-3 activation, cytochrome c release, and down-regulation of the Bcl-2 gene) occurred at etoposide concentrations below 176 μM. Therefore, the hepatocytes appear to be protected in some way against etoposide-induced apoptosis.

Ifosfamide is commonly coadministered with etoposide in combination anticancer chemotherapies. Ifosfamide was included in the treatment regimens for pediatric patients in our previous pharmacokinetic studies because all of the patients had primary solid tumors (four patients with Ewing’s sarcoma, two patients with Hodgkin’s disease and one patient with non-Hodgkin’s lymphoma) that are routinely treated with etoposide/ifosfamide-containing regimens (Pang et al., 2001; Zheng et al., 2004). It has been shown that ifosfamide (50 μM) significantly increases CYP3A-dependent testosterone 6β-hydroxylation in human hepatocytes (Chang et al., 1997). This finding suggests that it is most likely the concomitant ifosfamide treatment rather than etoposide that results in the increased etoposide catechol concentrations observed in patients’ plasma during the 5-day regimens (Pang et al., 2001; Zheng et al., 2004). The finding that etoposide did not induce CYP3A4 protein level in human hepatocytes is in contrast to the report that etoposide can up-regulate luciferase gene expression under the control of the 7.8-kb 5′-flanking region of CYP3A4 gene in transgenic mice and NIH 3T3 cells (Schaeutz et al., 2002). It is possible that there is a CYP3A4 repressor(s) in human hepatocytes, which can be removed during the initiation of P450 induction. This repressor(s) may be either absent in transgenic mice and NIH 3T3 cells or, alternatively, may bind to a region distal from the CYP3A4 7.8-kb 5′-flanking sequence.

In summary, CYP3A4 plays the major role in etoposide O-demethylation, and CYP3A5 may contribute significantly to this metabolism at clinically relevant free etoposide concentrations. However, etoposide is unable to up-regulate CYP3A4 expression in human hepatocytes and has only a minor effect on up-regulation of CYP3A5. Therefore, the increased formation of etoposide catechol during etoposide therapy (Pang et al., 2001; Zheng et al., 2004) is most likely due to other drugs such as ifosfamide that are part of the dosing regimen. These results indicate that study of genetic variation in not only CYP3A4 but also CYP3A5 in relation to leukemogenic risk is warranted.

Acknowledgments. We are grateful to the Cell Center in University of Pennsylvania for nucleotide sequencing and PCR primer synthesis, as well as the Molecular Biology Core in the Center for Molecular Studies in Digestive and Liver Disease, University of Pennsylvania for use of the API 7000 DNA sequence detection system.

References


Chang TK, Yu L, Maurel P, and Waxman DJ (1997) Enhanced cyclophosphamide and ifos-

Address correspondence to: Dr. Ian A. Blair, 854 BRB II/III, Cancer Pharmacology, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104-6160. E-mail: ian@spirit.gcrc.upenn.edu