Potential Contribution of Cytochrome P450 2B6 to Hepatic 4-Hydroxycyclophosphamide Formation In Vitro and In Vivo^S

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ABSTRACT:

Results from retrospective studies on the relationship between cytochrome P450 (P450) 2B6 (CYP2B6) genotype and cyclophosphamide (CY) efficacy and toxicity in adult cancer patients have been conflicting. We evaluated this relationship in children, who have faster CY clearance and receive different CY-based regimens than adults. These factors may influence the P450s metabolizing CY to 4-hydroxycyclophosphamide (4HCY), the principal precursor to CY's cytotoxic metabolite. Therefore, we sought to characterize the in vitro and in vivo roles of hepatic CYP2B6 and its main allelic variants in 4HCY formation. CYP2B6 is the major isozyme responsible for 4HCY formation in recombinant P450 Supersomes. In human liver microsomes (HLM), 4HCY formation correlated with known phenotypic markers of CYP2B6 activity, specifically formation of (S)-2-ethyl-1,5-dimethyl-3,3-diphenyl pyrrolidine and hy-

droxybupropion. However, in HLM, CYP3A4/5 also contributes to 4HCY formation at the CY concentrations similar to plasma concentrations achieved in children (0.1 mM). 4HCY formation was not associated with CYP2B6 genotype at low (0.1 mM) or high (1 mM) CY concentrations potentially because CYP3A4/5 and other isozymes also form 4HCY. To remove this confounder, 4HCY formation was evaluated in recombinant CYP2B6 enzymes, which demonstrated that 4HCY formation was lower for CYP2B6.4 and CYP2B6.5 compared with CYP2B6.1. In vivo, CYP2B6 genotype was not directly related to CY clearance or ratio of 4HCY/CY areas under the curve in 51 children receiving CY-based regimens. Concomitant chemotherapy agents did not influence 4HCY formation in vitro. We conclude that CYP2B6 genotype is not consistently related to 4HCY formation in vitro or in vivo.

Introduction

Cyclophosphamide (CY) is the cornerstone of curative chemotherapy regimens in over half of newly diagnosed pediatric cancer pa-

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tients. There is a pressing need to improve the efficacy and/or to decrease the toxicity of CY-based combination chemotherapy regimens. The current method of dosing CY based on body surface area or weight leads to considerable interpatient variability in systemic exposure to CY and its metabolites, including 4-hydroxycyclophosphamide (4HCY), the precursor to CY's primary cytotoxic metabolite phosphoramidate mustard (de Jonge et al., 2005a; McCune et al., 2009). The precise mechanism of 4HCY intracellular transport has not been evaluated; however, ABCC2 and ABCC4 are involved in the transport of CY and other metabolites (Qiu et al., 2004; Tian et al., 2005). Variability in 4HCY exposure, quantitated as area under the plasma concentration-time curve (AUC), may account for interpatient differences in the efficacy of CY.

Systemic exposure to 4HCY is determined by its formation by various cytochrome P450 (P450) isozymes, and potentially myeloperoxidase, along with its elimination by aldehyde dehydrogenases, glutathione S-transferases, and ATP-binding cassette transporters (Fig. 1) (de Jonge et al., 2005a). In vitro, CY is metabolized to 4HCY by various P450s, specifically CYP2A6, -2B6, -2C9, -2C18, -2C19, -3A4, and -3A5 (Roy et al., 1999; Xie et al., 2003; de Jonge et al., 2005a). 4HCY is transported into the cell, where it exists in equilib-

ABBREVIATIONS: CY, cyclophosphamide; P450, cytochrome P450; AUC, area under the curve; 4HCY, 4-hydroxycyclophosphamide; SNP, single nucleotide polymorphism; S-EDDP, (S)-2-ethyl-1,5-dimethyl-3,3-diphenyl pyrrolidine; HLM, human liver microsomes; PCR, polymerase chain reaction; TAO, troleandomycin; NBN, (+)-N-3-benzylirivanol; bp, base pair.

Cloning and Expression of CYP2B6 and Variants. To increase solubility and decrease membrane binding, the *CYP2B6* gene was modified similar to previously reported methods (Scott et al., 2001). The first 19 amino acids of the N terminus were removed, and residues 20 to 29 were replaced with the MAKTTSSGK sequence and constructed into the pCWori vector. A 6-histidine tag was inserted at the C terminus to aid in purification. No internal

TABLE 1

List of primers used in cloning of truncated CYP2B6 and variants

Primer Name	Sequence (5' to 3') ^a
2B6-truncation forward	CCATCGATCATATGGCTAAGAAGACTAG TAGTAAAGGAAACTCCACCAGGGCCCGCTCTG
2B6-truncation reverse	AGTCGACCGGGGAGGAGCGGATCTGGTATGTT
Internal SalI forward	GAAAAATCGCCATGGTGGACCCATT
CYP2B6*4 forward	CCCAGCGCCCCAGGGACCTCATCGAC
CYP2B6*6 forward	GACCCACCTTCTCTTCCATTCCATTACCGC
CYP2B6*5 forward	CAACATACCAGATCTGCTTCTGCCCGC

^a Base changes are underlined.

mutations were used to stabilize the protein. Human cDNA *CYP2B6* (Origene, Rockville, MD) was used as a template along with designed primers (Table 1) to form the truncated version of *CYP2B6*. The pCW2B6 plasmid was used as a template to create pCW2B6*4, pCW2B6*6, and pCW2B6*5. The plasmids were created using a specific set of primers and a QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) to mutate the base pairs (bp) of interest as shown in Table 1. The entire gene of *CYP2B6**1 and variants was sequenced to ensure that only the desired mutations, and no extraneous mutations, were introduced by PCR. DNA sequencing was performed at the Sequencing Facility in the Department of Biochemistry, University of Washington. The pCW2B6 or variant plasmids were cotransformed with the Gro7 plasmid into *Escherichia coli* C41 cells under expression conditions similar to those described previously (Mitsuda and Iwasaki, 2006). Accurate mass analysis of CYP2B6.1 and variants was performed on a Micromass high-definition mass spectrometer system (Waters, Milford, MA) equipped with a Quadrupole/Triwave/Orthogonal Acceleration Time of Flight tandem hybrid mass spectrometer using a previously published procedure (Cheesman et al., 2003). For wild type and variants, the *m/z* experimentally obtained was within 4 Da of calculated mass.

Purification of CYP2B6 and Variants. Protein purification was performed following literature procedure (Cheesman et al., 2003) with the following changes. After the high-speed centrifugation, the protein supernatant was loaded onto a Ni²⁺ NTA superflow column (QIAGEN, Valencia, CA) and after elution was dialyzed against buffer I (three times) (100 mM KPi, pH 7.4, 20% glycerol, and 50 mM KCl) and stored at -80°C at concentrations between 3 and 10 μ M. CO-difference spectra (Omura and Sato, 1964) and sodium dodecyl sulfate polyacrylamide gel electrophoresis were used to determine concentration and protein purity. A pyridine hemochromogen assay was performed and compared with CO-difference spectra to determine percentage of heme incorporation in wild type and variants.

Human Liver Microsomes. HLM samples ($n = 64$) were obtained from the University of Washington School of Pharmacy Human Tissue Bank. HLM were prepared as described previously (Lin et al., 2002). Five of the 64 HLM were obtained from donors ≤ 10 years of age, but only one HLM was available for analysis of 4HCY formation. CYP2B6 and CYP3A4/5 protein content in HLM ($n = 64$) and genotype ($n = 59$) had been completed prior to this investigation; the library is representative of the most common allelic variants of *CYP2B6* found within the white population (Totah et al., 2008). Because of the high protein concentration necessary to characterize 4HCY formation, Michaelis-Menten and inhibition experiments were conducted in pooled HLM, which were abundantly available.

Stability of 4HCY in Incubations. 4HCY was formed by the reduction of 4-hydroperoxycyclophosphamide in 50 mM KPi, pH 7.4. A stock solution of 4HCY (100 μ M) was made by adding 4-hydroperoxycyclophosphamide and an equal amount of sodium thiosulfate allowed to react for 10 min at room temperature. After 10 min, a 20- μ l aliquot of the 4HCY stock solution was placed into a shaking water bath at 37°C containing buffer and CYP2B6 Supersomes (5 pmol). NADPH was added after a 5-min preincubation. The final concentration of 4HCY within the incubation was 10 μ M. An equal volume of methanol to quench was added to the incubation mixture at 0, 5, 20, and 30 min, and the 4HCY was stabilized as described below. To ensure that there was no loss of 4HCY during this 5-min preincubation, a shorter experiment was performed in 50 mM KPi, pH 7.4, at 37°C using the method of 4HCY formation described above. After 5 min, $96.4 \pm 3.5\%$ remained compared with the 0-min control. Approximately 90% of the original 4HCY remained after a 30-min incubation at 37°C.

Inhibition of 4HCY Formation by Specific Chemical Inhibitors and Chemotherapeutics. Inhibition experiments were performed in quadruplicate. The final reaction volume for all inhibition experiments was 200 μ l. Specific inhibitors of CYP2B6 (1 μ M clopidogrel in HLM, 10 μ M in CYP2B6 Supersomes), CYP2C8 (1 μ M montelukast), CYP2C9 (10 μ M sulfaphenazole), CYP2C19 (5 μ M NBN), and CYP3A4/5 (100 μ M TAO and 1 μ M ketoconazole) were used to determine the contribution of each P450 to 4HCY formation in pooled HLM (Ren et al., 1997; Totah et al., 2008; Lee et al., 2010). For CYP2C8, CYP2C9, and CYP2C19, inhibition experiments were conducted using competitive conditions as presented below for the concomitant chemotherapeutic inhibition. In time-dependent inhibition experiments, clopidogrel and TAO were preincubated with pooled HLM (0.08 mg) at 37°C for 15 min with 1 mM NADPH before initiating the reaction with 0.1 mM CY. Inhibition experiments with all four inhibitors were performed similar to reactions with TAO and clopidogrel alone and in combination. All reactions were quenched after a 10-min incubation with 200 μ l of methanol. The 10-min incubation time was used to prevent extensive metabolism of montelukast (Walsky et al., 2005).

To determine whether concomitant medications competitively inhibited CY metabolism in HLM, chemotherapeutics were used at their maximum plasma concentration ($C_{\max}$): actinomycin, cytarabine, doxorubicin, fludarabine, 6-mercaptopurine, topotecan, and vincristine. Fludarabine was included because of its increasing concomitant use with CY in children undergoing a hematopoietic cell transplant; the remaining chemotherapeutics were included because they were administered to children in the pharmacokinetic study (see below). Clopidogrel, used as a competitive inhibitor of CYP2B6, served as a positive control (Talakat et al., 2009).

Inhibition experiments were performed with pooled HLM (0.08 mg) or CYP2B6 Supersomes (5 pmol) in 50 mM KPi buffer (pH 7.4, 200 μ l). CY was added to the tubes at a concentration of 0.1 mM, and the reaction mixture was equilibrated at 37°C for 5 min. NADPH (1 mM) was used to initiate the reaction, which was quenched with 200 μ l of methanol after 30 min (HLM) or 10 min (Supersomes). The concentration of organic was $\leq 0.2\%$ for samples dissolved in acetonitrile and was $\leq 0.1\%$ for DMSO. Samples were then prepared and quantitated using the method described below.

4HCY concentrations were determined using the method of de Jonge et al. (2005b) with slight modifications. 4HCY was analyzed as the semicarbazone derivative, which was formed by adding 2 M semicarbazide (40 μ l) and 4 M HCl (20 μ l) to the quenched reaction tube. After 10 min, 4 M NaOH (20 μ l) and 4-hydroxycyclophosphamide-*d*₄ were added, and tubes were centrifuged at 20,800g for 5 min. For analysis of all samples, 5 μ l of supernatant was injected onto a Zorbax SB-C18 Extend column (50 $\times$ 2.1 mm, 3.5 μ m) (Agilent Technologies, Santa Clara, CA) and analyzed using high-performance liquid chromatography (Agilent 1100 Series LC) and electrospray ionization mass spectrometry (Agilent G1946A MSD) in the positive mode. The fragmentor and capillary voltage were optimized to 100 and 2000 V, respectively; the drying gas flow rate was 12 l/min with a temperature of 350°C, and the nebulizer pressure was 35 psi. The mobile phase consisted of 1 mM ammonium acetate (A), pH 6.3, and acetonitrile (B) with a flow rate of 0.4 ml/min. The initial percentage of 12% B was held for 2 min, increased to 25% B over 1 min, decreased back to 12% over 1 min, and held constant for 2 min. The lower limits of quantitation and detection for 4HCY were 7.5 and 2.5 ng/ml, respectively. The interday variability for low (15 ng/ml) and high (1000 ng/ml) quality controls was 13.0 and 5.8%, respectively; the intraday variability was 6.3 and 3.3% for low- and high-quality control samples, respectively. 4HCY and the internal standard, *d*₄-4HCY, were detected as their respective sodium adducts: [Na⁺C₈H₁₈Cl₂N₅O₃P] *m/z* 356 and [Na⁺C₈H₁₄D₄³⁷Cl₂N₅O₃P] *m/z* 362.

Michaelis-Menten constants (K_m and $V_{\max}$) for CY were determined with recombinant enzymes under conditions that were linear with respect to time (5–60 min) and protein concentration (1–200 pmol/ml). Reconstituted enzyme systems were prepared by mixing CYP2B6 or variants (10 pmol), rat P450 reductase (20 pmol), cytochrome *b*₅ (10 pmol), and 40 μ g L- α -dilauryl-sn-glycero-3-phosphocholine micelles on ice for 20 min. Reconstituted enzyme was diluted to the final volume of 200 μ l with 50 mM KPi, pH 7.4, followed by the addition of CY (0.1–20 mM). The final concentration of methanol in the reaction mixtures was 0.5%, a concentration known to have minimal effects on CYP2B6 activity (Vuppugalla et al., 2007). After equilibration in a shaking

water bath at 37°C for 5 min, NADPH (1 mM) was used to initiate the 10-min incubation, which was quenched as described above.

Phenotypic Markers of CYP3A4/5 and CYP2B6 Activity in HLM. CYP3A4/5 catalyzed formation of 1-hydroxymidazolam in the HLM bank was determined in a previous study (Lin et al., 2002). The formation of (*S*)-2-ethyl-1,5-dimethyl-3,3-diphenyl pyrrolidine (*S*-EDDP) from *S*-methadone and the hydroxylation of bupropion were both evaluated as phenotypic markers of CYP2B6 activity in HLM. Formation of *S*-EDDP from *S*-methadone in HLM with a known CYP2B6 genotype and protein content has been reported previously (Totah et al., 2008). Incubations measuring hydroxybupropion were conducted in 100 mM KPi, pH 7.4, at 37°C after 5 min of preincubation with HLM (0.02 mg) and bupropion (75 μ M). Reactions were initiated with a reduced NADPH regenerating system that contained 10 mM glucose 6-phosphate, 1 mM β -NADP, 1 U/ml glucose 6-phosphate dehydrogenase, and 5 mM MgCl_2 . Experiments were performed in duplicate unless stated otherwise. After 30 min, reactions were quenched with an equivalent volume of acetonitrile containing hydroxybupropion-*d*₆ as the internal standard. Linear relationships were determined between 4HCY and formation of 1-hydroxymidazolam, hydroxybupropion, or *S*-EDDP to identify the role of CYP3A4/5 and CYP2B6 in catalysis at 0.1 mM CY.

4HCY Formation in Expressed Enzymes and HLM. Incubations were carried out with 0.1 or 1 mM CY in pooled HLM. Incubations were performed in disposable 2-ml microcentrifuge tubes containing 50 mM KPi, pH 7.4, and either 0.08 mg of human liver microsomal protein or 5 pmol of Supersomes in a final volume of 200 μ l. Samples were placed in a shaking water bath at 37°C and preincubated with CY for 5 min; the reaction was then initiated with 1 mM NADPH (10% of the volume). Reactions were incubated for either 30 min (HLM) or 10 min (Supersomes) and terminated with an equal volume of ice-cold methanol.

Prior incubations were conducted to ensure that all experiments were performed under linear conditions for both time and protein content. In pooled HLM incubated with 0.02 to 20 mM CY, the Michaelis-Menten parameters for CY were a $K_m = 2.38$ mM and $V_{max} = 1.77$ nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ (Supplemental Fig. 1).

Patient Population. Fifty-one children receiving their first cycle of a CY-based combination chemotherapy regimen were enrolled into the pharmacokinetic study. The median age was 5.5 years (range, 1.1–17.5); there were 30 male and 21 female participants. Children enrolled in this study were diagnosed with acute lymphoblastic leukemia ($n = 31$), Ewing sarcoma ($n = 12$), lymphoma ($n = 2$), neuroblastoma ($n = 2$), rhabdomyosarcoma ($n = 2$), or other malignancies ($n = 2$). The majority of children received CY for a single day as part of the following regimens: 17 children received consolidation (1000 mg/m² CY for 1 day, 75 mg/m² cytarabine for 4 days, and 60 mg/m² 6-mercaptopurine for 14 days), 16 children received delayed intensification (1000 mg/m² CY for 1 day, 75 mg/m² cytarabine for 4 days, and 60 mg/m² thioguanine for 14 days), 15 children received VDC (1200 mg/m² CY for 1 day, 1.5 mg/m² vincristine for 1 day, and 37.5 mg/m² per day doxorubicin for 2 days), and one child received VAC (1200 mg/m² CY, 1.5 mg/m² vincristine, and 0.045 mg/kg actinomycin; all were administered for 1 day). The abbreviations VAC and VDC are both based on the first letter of each antineoplastic in their regimen. The two children with neuroblastoma received fractionated CY at a dose of 400 mg/m² per day in conjunction with topotecan (1.2 mg/m² per day) for 5 days. All participants received similar antiemetics, antibiotics, and antifungals per institutional standard practice guidelines. Two children received fluconazole concomitant with CY. The Seattle Children's Hospital Institutional Review Board approved the study, and all participants' parents gave informed consent before participation. In addition to written informed consent from their parents or guardians, children aged 14 to 17 years gave written assent.

Pharmacokinetic Analysis of CY and Metabolites. Pharmacokinetic samples were drawn immediately before CY administration, after completion of the 30-min infusion, and at 2.5, 4.5, 6.5, 22.5, and 24 h after the start of the first CY dose. The sample processing and analysis procedures have been published previously (McCune et al., 2009). In brief, blood was drawn into tubes containing either phenylhydrazine, for analysis of 4HCY, or ethylenediaminetetraacetic acid, for analysis of CY and carboxyethylphosphoramidate mustard. The tubes were inverted three to four times, stored at 4°C for a maximum of 1 h, and centrifuged. Plasma was removed and stored at -70°C

within 1 h of sample collection. All samples were shipped on dry ice to our laboratory within 3 months of collection. Plasma concentrations were determined for CY and its metabolites using a previously reported methodology; 4HCY concentrations were quantitated separately (McCune et al., 2009). A noncompartmental model was used to calculate the AUC from time 0 to infinity after the first CY dose using WinNonlin version 2.0 (Pharsight, Mountain View, CA).

Genotyping of CYP2B6. Genomic DNA was isolated from a 10-ml blood sample using Gentra Purgene DNA Purification kits (Gentra Systems, Inc., Minneapolis, MN). All methods were validated using a panel of 96 Utah Centre d'Etude du Polymorphisme Humain (Paris, France) DNA samples; genotype and allele frequencies were consistent with reported results. The quality and quantity of DNA in each sample were determined using the absorbance ratio of 260 nm/280 nm.

Genotyping was performed for CYP2B6*4 allele (K262R, rs2279343) using the primers and protocol described in a previous report (Jacob et al., 2004). In brief, PCRs were performed with 50 ng of genomic DNA, 1.5 mM MgCl_2 , 100 μ M nucleotides, 100 μ M of primers, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in a total volume of 20 μ l. The conditions in a PE 9700 Thermocycler were as follows: one cycle for 5 min at 94°C, five cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, and a final extension of 72°C for 10 min. The resulting 1474-bp product was digested with *StyI* (New England Biolabs, Beverly, MA), and the fragments generated were analyzed by gel electrophoresis and ethidium bromide staining. Fragments of either 56/340/1078 or 56/171/340/907 bp were produced, indicating the presence of the variant (G allele, 262R) or wild-type (A allele, K262) allele, respectively. Patient samples were genotyped for the CYP2B6*5 allele (R487C, rs3211371, assay ID C 30634242_40) and CYP2B6*6 SNP (Q172H, rs37445274, assay ID C 7817765_60) using TaqMan SNP Genotyping Assays (Applied Biosystems). The reaction mixture for both assays consisted of 4 ng of genomic DNA, 20 $\times$ drug metabolism genotyping assay mix, and 2 $\times$ Universal PCR Master Mix in a total volume of 5 μ l. Real-time PCR was done on the 7900HT system using standard Taqman cycling conditions. Allelic discrimination analysis was carried out using the Sequence Detection System (version 2.3; Applied Biosystems). Patients that carried the variant allele for both K262R (rs2279343) and Q172H (rs37445274) were designated as CYP2B6*6, consistent with the definition of this allele (<http://www.cypalleles.ki.se/cyp2b6.htm>). Patients carrying the wild-type allele for each of the three SNPs assayed (K262R rs2279343; R487C rs3211371; Q172H rs37445274) were designated as CYP2B6*1.

Data Analysis. Statistical analysis of kinetic data was performed using GraphPad Prism version 5.2 (GraphPad Software Inc., San Diego, CA). 4HCY formation versus CY concentration data were analyzed by a Michaelis-Menten model using nonlinear regression analysis for the determination of K_m and V_{max} . The intrinsic clearance was calculated using V_{max}/K_m . The total normalized rate of 4HCY formation at 0.1 mM CY is determined as reported previously (Rodrigues, 1999). The 4HCY formation determined in Supersomes was multiplied by the average content (in picomoles per milligram) for each specific P450 to determine the normalized rate of 4HCY formation. The P450 hepatic average content used was either determined from the literature (CYP2C9, 88.5 pmol/mg; and CYP2C19, 17.8 pmol/mg) or from the average content in our HLM bank (CYP2B6, 20.7 pmol/mg; CYP2C8, 31.2 pmol/mg; CYP3A4, 80.1 pmol/mg; CYP3A5, 27.5 pmol/mg) (Lasker et al., 1998; Lin et al., 2002; Totah et al., 2008; Naraharisetti et al., 2010). The percentage of total normalized rate of 4HCY formation was determined by dividing the normalized rate for each P450 by the sum of all rates and multiplying by 100%.

Linear regression was used to determine the correlations between CYP2B6 protein content and age, hydroxybupropion and 1-hydroxymidazolam formation, 4HCY and 1-hydroxymidazolam formation, 4HCY and hydroxybupropion formation, and 4HCY and *S*-EDDP formation. In all cases, a Spearman rank-order correlation coefficient was used to determine the significance of the association between these variables. A nonparametric Mann-Whitney *U* test was used to determine a significant association between CYP2B6 protein content and age. A one-way analysis of variance using a Kruskal-Wallis test was performed to determine statistically significant differences between CYP2B6 genotype groups. For all data analyses, a *p* value less than 0.05 was regarded as statistically significant.

Results

4HCY Formation in Supersomes and Normalized Reaction Rate Calculation.

A panel of cDNA-expressed P450 isozymes (Supersomes) with coexpressed human reductase and cytochrome b_5 was screened to determine the contribution of each enzyme to the formation of 4HCY. At 0.1 mM CY, CYP2B6 had the greatest contribution to 4HCY formation. In Supersomes, the formation of 4HCY by CYP2B6 was 7-fold higher (13.3 ± 0.7 pmol 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$) than the other main contributors: CYP3A4 (2.02 ± 0.07 pmol 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$), CYP2C19 (1.83 ± 0.04 pmol 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$), CYP3A5 (1.10 ± 0.02 pmol 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$), CYP2C9 (0.370 ± 0.015 pmol 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$), and CYP2C8 (0.115 ± 0.004 pmol 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$). CYP2E1 and CYP2J2 had negligible 4HCY formation (0.057 ± 0.004 pmol and 0.052 ± 0.005 4HCY $\cdot$ min $^{-1}$ $\cdot$ pmol P450 $^{-1}$). For a more accurate assessment of the contribution of each isozyme to 4HCY formation, rates obtained in Supersomes were normalized to average hepatic content (Rodrigues, 1999) (Fig. 2A, open bars). The average total normalized rates and S.D.s for CYP3A4 and CYP3A5 were combined after the initial calculation. CYP2B6 remained a major contributor to 4HCY formation ($51.4 \pm 2.6\%$) followed by CYP3A4/5 ($35.8 \pm 1.1\%$). Both CYP2C9 and CYP2C19 showed equal contribution (6.11 ± 0.25 and $6.07 \pm 0.13\%$, respectively), whereas CYP2C8 ($0.67 \pm 0.03\%$) had a negligible contribution.

Inhibition of 4HCY Formation by Specific Chemical Inhibitors.

Chemical inhibitor experiments were conducted to further elucidate CYP2B6, CYP3A4/5, CYP2C9, and CYP2C19 on 4HCY formation rate at a low CY (0.1 mM) concentration. These experiments were conducted in Supersomes and HLM (Fig. 2B). Clopidogrel, a CYP2B6 inhibitor, had the largest effect on 4HCY formation ($52.2 \pm 5.6\%$ remaining activity). The two CYP3A4/5 inhibitors, ketoconazole and TAO, had a similar effect on 4HCY formation (73.1 ± 5.2 and $60.4 \pm 2.6\%$ remaining activity, respectively). However, CYP2C inhibitors had a less dramatic effect ranging from $80.2 \pm 1.5\%$ remaining activity for sulfaphenazole to $95.0 \pm 6.8\%$ remaining activity for montelukast. After incubation with both TAO and clopidogrel, 4HCY formation was only $32.2 \pm 4.4\%$ of the control.

For validation of the percentage of remaining activity experiments in the pooled HLM, the total normalized rates were compared with the

inhibition of 4HCY formation with specific chemical inhibitors in Supersomes. The percentage of total normalized rate and the percentage of inhibition were similar for each isozyme (Fig. 2A), with the exception that sulfaphenazole, the CYP2C9-specific inhibitor, had a greater percentage of inhibition ($19.8 \pm 1.5\%$; Fig. 2A, filled bars) than that predicted by the percentage of total normalized rate. Thus, CYP2C9 may have a greater impact on 4HCY formation than suggested by the percentage of remaining activity in Fig. 2B.

4HCY Formation in Matched HLM Samples. To further elucidate the effect of CYP2B6 on the rate of 4HCY formation in HLM, a panel of 10 paired HLM samples with a predetermined CYP2B6 genotype was assessed. CYP3A4/5 protein content was matched in each pair, whereas CYP2B6 protein content varied (6.84–80.6 pmol/mg) within each pair, and the formation of 4HCY at 0.1 mM CY was determined. Within the five pairs, the HLM with lower CYP2B6 content showed lower 4HCY formation (Table 2) at similar CYP3A4/5 content. However, 4HCY formation was higher in those HLM with high CYP3A4/5, which suggests CYP3A4/5 is a critically important enzyme involved in 4HCY formation.

Association of 4HCY Formation with Phenotypic Markers of CYP2B6 and CYP3A4/5 in HLM. The involvement of CYP3A4/5 and CYP2B6 in 4HCY formation was assessed using a correlation analysis in the HLM bank and specific phenotypic markers for both isozymes. The associations between 4HCY formation and 1-hydroxymidazolam formation, a marker of CYP3A4/5 activity, and the formation of S-EDDP and hydroxybupropion, two established phenotypic markers of CYP2B6 activity, were evaluated (Hesse et al., 2004; Huang et al., 2004; Totah et al., 2008). Metabolite formation was determined in HLM at 8 μ M midazolam, the reported substrate K_m for CYP2B6 (75 μ M bupropion), or at a therapeutic concentration (1 μ M S-methadone or 0.1 mM CY) (Lin et al., 2002; Hesse et al., 2004; Totah et al., 2008; McCune et al., 2009). Formation of hydroxybupropion and 1-hydroxymidazolam was correlated (Fig. 3A), in agreement with previous data that CYP2B6 and CYP3A4 protein content in HLM was significantly associated (Totah et al., 2008). There was a significant correlation between 4HCY formation and all phenotypic markers. Significant associations between 1-hydroxymidazolam and hydroxybupropion formation (Fig. 3A; $r = 0.58$, $p < 0.0001$, $n = 47$), 4HCY and 1-hydroxymidazolam formation (Fig. 3B; $r = 0.69$, $p <$

FIG. 2. Inhibition of cyclophosphamide 4-hydroxylation by specific chemical inhibitors and the total normalized rate assessed in pooled human liver microsomes. The rate of 4HCY formation was determined in Supersomes (5 pmol) after a 10-min incubation with 0.1 mM CY. A, the percentage of total normalized rate (as determined in *Materials and Methods*) is plotted and compared with the percentage of inhibition obtained from specific chemical inhibition. As described under *Materials and Methods*, 4HCY formation in pooled HLM (0.08 mg) was measured after 10-min incubation. The concentration of specific chemical inhibitors were clopidogrel (1 μ M), montelukast (1 μ M), NBN (5 μ M), sulfaphenazole (10 μ M), TAO (100 μ M), and ketoconazole (1 μ M). The term "All four" is used to abbreviate the addition of clopidogrel, NBN, sulfaphenazole, and TAO. B, data are displayed as percentage reduction in control (no inhibitor) activity. Each data point is mean $\pm$ S.D. of four replicates.

TABLE 2
CY 4-hydroxylation in HLM matched for CYP3A content

HLMs (CYP2B6 Genotype) ^a	Pair	Matched Pair Description	CYP450 Enzyme Content		Formation of 4HCY $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$
			CYP3A	CYP2B6	
			pmol/mg		
HLM 141 (*1/*1)	A	High 3A, high 2B6	381	69.2	235
HLM 144 (*1/*6)		High 3A, moderate 2B6	351	27.0	172
HLM 124 (*1/*6)	B	High 3A, high 2B6	176	49.5	75.9
HLM 148 (*6/*6)		High 3A, low 2B6	171	12.2	29.6
HLM 149 (*1/*6)	C	Moderate 3A, moderate 2B6	154	31.8	122
HLM 166 (*1/*1)		Moderate 3A, low 2B6	146	14.0	28.5
HLM 139 (*1/*4)	D	Low 3A, high 2B6	69.6	80.6	66.0
HLM 120 (*1/*1)		Low 3A, low 2B6	71.2	15.5	27.6
HLM 142 (*1/*4)	E	Low 3A, moderate 2B6	34.8	29.4	68.4
HLM 164 (*1/*6)		Low 3A, low 2B6	36.3	6.84	21.9

^a Pairs are ordered by decreasing CYP3A content; CYP2B6 content was varied to determine CY 4-hydroxylation in high and low CYP2B6 content samples. Each value represents the mean of two replicates.

0.0001, $n = 37$), 4HCY and hydroxybupropion formation (Fig. 3C; $r = 0.96$, $p < 0.0001$, $n = 31$), and 4HCY and *S*-EDDP formation (Fig. 3D; $r = 0.68$, $p < 0.01$, $n = 13$) were found.

Effects of CYP2B6 Genotype on 4HCY Formation. The effect of CYP2B6 genotype on CY metabolism was investigated by measuring 4HCY formation in a sample set of HLM using two

concentrations that reflected the CY plasma C_{max} in children (0.1 mM) or were similar to the reported K_m of CY with CYP2B6 (1 mM) (Huang et al., 2000; Xie et al., 2003). A Kruskal-Wallis test showed no significant association between 4HCY formation and CYP2B6 genotype at CY concentrations of 0.1 mM (Fig. 4A; $p = 0.43$, $n = 39$) and 1 mM (Fig. 4B; $p = 0.42$, $n = 39$). Supplemental

FIG. 3. Association of cyclophosphamide 4-hydroxylation with phenotypic markers of CYP2B6 and CYP3A4 activity in human liver microsomes. Correlation of the hydroxybupropion formation with 1-hydroxymidazolam formation (A; $r = 0.58$, $p < 0.0001$, $n = 47$). B–D, correlation of 4HCY formation with 1-hydroxymidazolam formation (B; $r = 0.69$, $p < 0.0001$, $n = 37$), hydroxybupropion formation (C; $r = 0.96$, $p < 0.0001$, $n = 31$), or *S*-EDDP formation (D; $r = 0.68$, $p < 0.01$, $n = 13$). The substrate concentrations used were 0.1 mM CY, 8 μM midazolam, 75 μM bupropion, and 1 μM *S*-methadone. Each data point is the mean of duplicate samples.

Fig. 4. Variation of cyclophosphamide 4-hydroxylation by *CYP2B6* genotype at low (0.1 mM; A) and high (1 mM; B) CY concentrations in human liver microsomes. A, 4HCY formation using 0.1 mM CY ($p = 0.43$). B, 4HCY formation using 1 mM CY ($p = 0.42$). Each data point is the mean of duplicate samples with the horizontal line representing the median value for each *CYP2B6* genotype. Each reaction mixture contained 0.08 mg of HLM and was incubated for 30 min at 37°C. Incubations performed with HLM. HLM from donors <18 years of age ($n = 2$) are denoted by open triangles.

Fig. 2 shows that age, gender, and *CYP2B6* genotype had no effect on *CYP2B6* protein content in these HLM.

Unfortunately, there was a small number of *CYP2B6**1/*4 ($n = 3$), *1/*5 ($n = 2$), *1/*7 ($n = 4$), and *6/*6 ($n = 1$) genotypes within the HLM bank, which made it difficult to draw strong conclusions about the influence of *CYP2B6* genotype on 4HCY formation. Compared with wild type, average 4HCY formation was reduced by 50% in the *CYP2B6**1/*4 ($n = 3$) population in experiments with 0.1 and 1 mM CY (Fig. 4).

4HCY Formation in Recombinant *CYP2B6* and Most Common Variants. The metabolism of CY was fully characterized in *CYP2B6*.1 (wild type) and most common variants using recombinant engineered proteins expressed and purified using an *E. coli* expression system (Fig. 5). The recombinant proteins contained only modifications to the N- and C-terminal regions to increase their solubility, hydrophilicity, and aid in purification. No further mutations were introduced within the coding region, and this was confirmed by full sequencing of the plasmid prior to expression. The intrinsic clearance for all recombinant enzymes was determined using $V_{\max}/K_m$. A Kruskal-Wallis test showed no significant difference ($p = 0.39$) in intrinsic clearance between *CYP2B6**1 and variants. Intrinsic CY clearance for *CYP2B6*.6 was ~30% higher than *CYP2B6*.1 (Table 3; Fig. 5), but both *CYP2B6*.4 and *CYP2B6*.5 displayed a reduction in intrinsic CY clearance compared with wild type. In fact, *CYP2B6*.5 intrinsic clearance was reduced by 50% and *CYP2B6*.4 by 30%. Supplemental Table 1 shows the intrinsic clearance of hydroxybupropion in *E. coli*-expressed *CYP2B6* recombinant enzymes and *CYP2B6* Supersomes.

Effect of Coadministered Chemotherapeutics on 4HCY Formation in Pooled HLM and *CYP2B6* Supersomes. The effect of commonly coadministered chemotherapeutics on *CYP2B6*-mediated 4HCY formation was examined. No coadministered chemotherapeutic reduced 4HCY formation in pooled HLM or *CYP2B6* Supersomes. In comparison, clopidogrel reduced 4HCY formation to $22.9 \pm 2.5\%$ of control activity in pooled HLM and $1.35 \pm 0.95\%$ of control activity in *CYP2B6* Supersomes (Supplemental Fig. 3).

Effect of *CYP2B6* Genotype on the Pharmacokinetics of CY in Pediatric Patients Receiving Various Chemotherapeutic Regimens. Children ($n = 51$, 30 males) aged 1.1 to 17.5 years receiving

CY-based chemotherapy (see *Materials and Methods*) were genotyped for *CYP2B6**1, *CYP2B6**4, *CYP2B6**5, and *CYP2B6**6 (Fig. 6). The ethnicity of this population was 39 white persons, two African Americans, three Hispanics, two Asians, and five persons of unknown race. All *CYP2B6*-observed genotypes were in Hardy-Weinberg equilibrium and were consistent with what has been previously reported in white populations (Lang et al., 2001). *CYP2B6* genotypes in this population were as follows: *CYP2B6**1/*1 ($n = 22$), *CYP2B6**1/*6 allele ($n = 19$), *CYP2B6**6/*6 ($n = 1$), *CYP2B6**1/*5 ($n = 8$), and *CYP2B6**4/*6 ($n = 1$).

CY clearance was determined as described previously (McCune et al., 2009). The median (range) of the maximum plasma concentration was 126 μM (111–141 μM) for the two children receiving 400 mg/m² CY and 318 μM (187–387 μM) for the remaining children receiving >1000 mg/m² CY. Median CY clearance was $2.20 \text{ l} \cdot \text{h}^{-1} \cdot \text{m}^{-2}$ (1.39 – $4.30 \text{ l} \cdot \text{h}^{-1} \cdot \text{m}^{-2}$), and the median ratio of 4HCY/CY AUCs was 0.044 (0.017–0.137). Correlations were determined between *CYP2B6* genotype and CY clearance (Fig. 6A) or the ratio of 4HCY/CY AUCs (Fig. 6B). A Kruskal-Wallis test was applied to determine whether differences between groups were significant: no statistically significant association was observed between *CYP2B6* genotype and CY clearance ($p = 0.57$) or the ratio of 4HCY/CY AUCs ($p = 0.37$).

Discussion

This study sought to thoroughly investigate the roles of *CYP2B6* and *CYP2B6* genetic variation in two systems: the in vitro 4-hydroxylation of CY and CY pharmacokinetics in pediatric patients. Our key findings were that *CYP2B6* and *CYP3A4/5* are involved in 4HCY formation in vitro at a low CY concentration of 0.1 mM, which is pharmacologically relevant to children, that 4HCY formation is not affected by chemotherapy agents often coadministered to children, and that *CYP2B6* genotype is not clearly associated with 4HCY formation in either HLM or children receiving CY.

CYP2B6 is the predominant P450 involved in 4HCY formation at low CY concentrations of 0.1 mM based on in vitro experiments with Supersomes and after normalizing rates with respect to rela-

TABLE 3
Kinetic parameters of 4HCY formation determined in reconstituted enzyme systems

Enzyme	Amino Acid Change	K_m^a mM	$V_{\max}^a$ $\text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$	Intrinsic Clearance ^b $\text{nl} \cdot \text{min}^{-1} \cdot \text{pmol P450}^{-1}$
<i>CYP2B6</i> .1	None	3.6 ± 0.1	75.4 ± 0.9	20.7
<i>CYP2B6</i> .4	K262R	3.5 ± 0.1	50.6 ± 0.6	14.4
<i>CYP2B6</i> .5	R487C	5.1 ± 0.4	54.6 ± 1.5	10.8
<i>CYP2B6</i> .6	K262R/Q172H	4.0 ± 0.1	117 ± 1	29.0

^a Mean $\pm$ S.D.

^b $V_{\max}/K_m$.

FIG. 5. Kinetic parameters of CY 4-hydroxylation in purified CYP2B6 enzymes. CY (0.1–20 mM) was incubated with reconstituted systems (10 pmol) containing CYP2B6.1 (●), CYP2B6.4 (■), CYP2B6.5 (▼), or CYP2B6.6 (▲) for 10 min at 37°C. Data points are mean $\pm$ S.D. of four replicates.

tive abundance of each P450 (Fig. 2A), which contrasts with data suggesting that CYP2C9 may predominate at these low concentrations (Ren et al., 1997). At the time, Ren et al. (1997) evaluated the role of P450 in 4HCY formation in HLM; a specific inhibitor of CYP2B6 was not available, and therefore the contribution of CYP2B6 could not be adequately determined. Notably, the relationship of plasma concentrations to hepatic concentrations is not known so it is assumed that the lower CY plasma concentrations observed in children translates to lower CY hepatic concentrations. In addition, our data also revealed greater 4HCY formation by CYP3A5, most likely due to inclusion of coexpressed cytochrome b_5 in recent batches of Supersomes that show an increase in their catalytic activity with other substrates. We evaluated the relative contributions, using specific chemical inhibitors, of CYP3A4/5 and CYP2B6 to 4HCY formation, because CYP3A4/5 catalyzes 4HCY formation more slowly but has greater hepatic expression compared with CYP2B6. In pooled HLM, a pool of 50 livers of mixed gender, both CYP3A4/5 and CYP2B6 contributed to 4HCY formation at 0.1 mM CY. A linear relationship between 4HCY formation and phenotypic markers of CYP3A4/5, and CYP2B6 activity, was also established (Fig. 3). Our results strongly indicate that CYP3A4/5 and CYP2B6 are involved in 4HCY formation at low CY concentrations.

Both nongenetic and genetic factors associated with CYP2B6 protein content in HLM (Supplemental Fig. 2) were performed by determining the relationship between CYP2B6 protein content and genotype, age, or gender. HLM from female donors had higher CYP2B6

content than those of male donors, but genotype and age were not significantly associated with CYP2B6 content. These results indicate our HLM bank provides a reliable model for the effect of *CYP2B6* genotype on 4HCY formation. However, one limitation of our work was that our HLM bank was predominantly obtained from adult donors.

To more comprehensively evaluate the effects of *CYP2B6* allelic variants, purified enzyme systems were used. Previous reports of the K_m in CYP2B6 Supersomes ranged between 1.36 and 1.89 mM (Huang et al., 2000; Xie et al., 2003), which is lower than the K_m (3.6 mM) obtained within our reconstituted enzyme system. The V_{max} (75.4 pmol $\cdot$ min $^{-1}$ $\cdot$ pmol $^{-1}$) obtained for 4HCY formation in our reconstituted system was lower than previous studies (Xie et al., 2003). Kumar et al. (2006) reported that differences between the kinetic parameters in Supersomes and reconstituted enzyme systems could be due to variation in the cytochrome b_5 and reductase concentrations in Supersomes. Michaelis-Menten parameters for CY were also evaluated in pooled HLM. K_m (2.38 mM) and V_{max} (1.77 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$) values obtained were within the range of literature values (Griskevicius et al., 2003; Helsby et al., 2010). However, the absence of a genotype-phenotype relationship for CYP2B6 would be consistent with CYP2B6 contributing to metabolite formation but not quantitatively the most important determinant of metabolite formation. It is also interesting that the Eadie-Hofstee plot indicated single enzyme kinetics, which suggests that even though multiple P450s are involved in 4HCY formation, they possess similar Michaelis-Menten parameters.

There have been previous reports suggesting that carriers of the *CYP2B6**6 allele have increased 4HCY formation in vitro (Xie et al., 2003). Within the HLM bank, there was no significant difference in the rate of 4HCY formation between *CYP2B6**1/*1 and *1/*6, but further analysis with purified CYP2B6.6 yielded a modest increase in the intrinsic clearance (V_{max}/K_m) of CY ($\sim 30\%$) compared with CYP2B6.1. In the purified enzyme systems, there was an observed reduction in the catalytic ability of the CYP2B6.4 variant. Although this trend was confirmed in the HLM bank, it did not reach statistical significance, potentially due to the limited number ($n = 3$) of *CYP2B6**1/*4 donors; alternatively, the other P450s involved in 4HCY formation may have obscured a *CYP2B6* genotype-4HCY formation phenotype relationship. The latter theory is supported by 51.3% inhibition of 4HCY formation in HLM by clopidogrel, a known CYP2B6 inhibitor. It is also possible that the effect of the *CYP2B6**4 variant on metabolism is substrate dependent (Rotger et al., 2007).

FIG. 6. CY clearance (A) and ratio of 4HCY/CY AUCs (B) by *CYP2B6* genotype in children receiving a conventional dose of CY. A, CY clearance (l $\cdot$ h $^{-1}$ $\cdot$ m $^{-2}$) for each *CYP2B6* genotype ($p = 0.57$). B, ratio of 4HCY/CY AUC for each *CYP2B6* genotype ($p = 0.37$). Pharmacokinetic samples were drawn immediately before CY administration, upon completion of the 30-min infusion, and at 2.5, 4.5, 6.5, 22.5, and 24 h after the start of the first CY dose. Children that received fluconazole concomitant with CY are denoted by open triangles. All others are denoted by filled circles. Genomic DNA was extracted from blood and genotyped for the most common *CYP2B6* variants as described under *Materials and Methods*. Horizontal lines represent the median value for each group; the Kruskal-Wallis test was used to determine significance.

We are the first to evaluate the relationship between *CYP2B6* genotype and the CY pharmacokinetic phenotype in children. However, our study is limited by the relatively small sample size ($n = 51$), the varying CY doses, and the heterogeneity of combination chemotherapy regimens. Pharmacokinetic studies of larger pediatric populations receiving a homogenous CY dose and regimen are desirable. The studies in adults that evaluate the *CYP2B6* genotype-CY pharmacokinetic phenotype relationship have been contradictory and inconclusive (Helsby and Tingle, 2011). The clearance of CY and the ratio of 4HCY/CY AUCs differed based on *CYP2B6* genotype in 103 Japanese patients receiving a conventional dose of CY (500–750 mg/m²). In particular, CY clearance was greater in the three patients with *CYP2B6**6/*6 genotype; however, one of the three patients had an appreciably higher clearance and may have skewed this analysis. Whereas the ratio of 4HCY/CY AUCs did not differ based on genotype, this ratio was lower in carriers of the single nucleotide polymorphisms of the *CYP2B6* gene, g.-2320T>C (rs7254579), g.-750T>C (rs4802101), g.15582C>T (rs4803419), or g.18492T>C (rs2279345) (Nakajima et al., 2007). In 29 white adults receiving CY (1000 mg/m²) with corticosteroids, the ratio of 4HCY/CY AUCs was 1.5-fold higher in the eight *CYP2B6* 516T (*CYP2B6**1/*9) (mean $\pm$ S.D. of ratio, 0.027 ± 0.020) carriers than in 21 *CYP2B6* 516 G/G homozygotes (*CYP2B6**1/*1), (mean $\pm$ S.D. of ratio, 0.017 ± 0.014) (Xie et al., 2006). A relationship was not observed between *CYP2B6* genotype and CY or 4HCY pharmacokinetics in 124 white persons receiving CY (1500 mg/m² per day for 4 days) with carboplatin and thiotepa (Ekhardt et al., 2008). However, thiotepa is a known inhibitor of CYP2B6 and thus would be expected to alter the pharmacokinetic phenotype (McCune et al., 2005). CY clearance was not associated with *CYP2B6* genotype (i.e., *2, *3, *4, *5, *6) in 60 adults receiving one of a variety of different CY combination chemotherapy regimens (mean $\pm$ S.D. of CY dose, 1008 ± 806 mg/m²). Unfortunately, 4HCY plasma concentrations were not available from that study (Timm et al., 2005). Therefore, the current literature regarding the relationship of *CYP2B6* genotype with CY or 4HCY pharmacokinetic phenotype has been contradictory in adults. More comprehensive genotype studies are desirable to evaluate for multifactorial reduced functional alleles (e.g., *CYP2B6*, *2C19*, and *3A5* genetic polymorphisms).

Although there is no data correlating *CYP2B6* genotype and CY pharmacokinetics in pediatric populations, there are studies in HIV-infected pediatric patients addressing the effects of polymorphisms on the CYP2B6 substrates efavirenz and nevirapine (Rotger et al., 2007; Saitoh et al., 2007). Homozygous and heterozygous *CYP2B6**6 carriers exhibit a marked decrease in the clearance of these drugs in vivo. We did not observe an association with *CYP2B6* and CY clearance or the ratio of 4HCY/CY AUCs (Fig. 6). Furthermore, *CYP2B6**6 genotype did not explain the large interindividual variability observed in the ratio of 4HCY/CY AUCs. Within the 19 *CYP2B6**6 heterozygotes, the ratio of 4HCY/CY AUCs still showed a 10-fold variation (Fig. 6B). This may be due to the many other P450s involved in CY hydroxylation and might nullify any phenotypic effects from the *CYP2B6**6 genotype, which is consistent with our in vitro data. Because children often receive different chemotherapy regimens than adults, we demonstrated in vitro that coadministered chemotherapeutics do not competitively inhibit CYP2B6, nor do they affect 4HCY formation (Supplemental Fig. 3). Thus, the lack of a *CYP2B6* genotype-pharmacokinetic phenotype association is unlikely to be caused by concomitant chemotherapy agents.

In conclusion, CYP3A4/5 and the highly polymorphic CYP2B6 are major contributors to 4HCY formation at the low (0.1 mM) CY concentrations that are pharmacologically relevant to children receiving a conventional dose of CY. Although 4HCY formation is altered

by common *CYP2B6* variants in the in vitro model of purified enzymes, there was no significant relationship between *CYP2B6* genotype and CY or 4HCY pharmacokinetics among children receiving a conventional dose of CY. Thus, we hypothesize this lack of a genotype-pharmacokinetic phenotype relationship is due to the other P450 isozymes involved in CY 4-hydroxylation. Additional work is ongoing to identify factors, genetic and nongenetic, associated with CY pharmacokinetics that may improve the efficacy of CY-based combination regimens in pediatric patients.

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Authorship Contributions

Participated in research design: Raccor, Claessens, Dinh, Park, Hawkins, McCune, and Totah.

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Performed data analysis: Raccor, McCune, and Totah.

Wrote or contributed to the writing of the manuscript: Raccor, Claessens, Dinh, Park, Hawkins, Thomas, Makar, McCune, and Totah.

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