OXAZAPHOSPHORINE METABOLISM BY THE HUMAN CYTOCHROME P450s AND SOME COMMONLY EXPRESSED P450 POLYMORPHIC VARIANTS

by

Diane M. Calinski

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in the University of Michigan
2013

Doctoral Committee:

Professor Paul F. Hollenberg, Chair
Professor Yoichi Osawa
Assistant Professor James M. Rae
Professor Lucy Waskell

To the millions of patients that have been treated with the anticancer agents cyclophosphamide and ifosfamide, and the thousands of researchers that are tirelessly striving to produce better cancer chemotherapies.

AKNOWLEDGMENTS

I would like to thank my mentor, Paul Hollenberg, for the endless support and guidance he has provided me during my time in his laboratory. I truly did not understand "mentorship" until I worked with Paul. He has not only taught me about cytochrome P450s, but also about patience, persistence, and the personal connections that are necessary to create fruitful, meaningful mentor-mentee relationships. I will forever use the lessons I have learned from our interactions, and I look forward to calling him my colleague.

The environment that Paul has created in his laboratory is designed for success. I would like to thank the laboratory members, Hsia-lien Lin and Dr. Haoming Zhang, for accepting me into the lab with open arms and providing me with anything I needed, be it purified proteins, experimental advice, or laughter.

Finally, I would like to thank my personal army of support. My parents Peter and Ann Calinski, for teaching (and constantly reminding me) what hard work is, and motivating me to see this task through with pride, grace, and my head held high. My siblings Joe, Karen, and Danny who have somehow managed to encourage me and lift up my spirits without letting any illusions of grandeur develop. The Walton-Swisher clan, for giving me a Michigan family, allowing me to steal their miracle child, and always championing me on

this journey. Lastly, my men Sam and Norm. Norm, you provide a particular sense of distraction and entertainment that only a 4-legged friend can. Sam, my biggest supporter, you probably didn't realize what you were taking on when you met me, but thank you for sticking with me, listening to all of my practice presentations, tolerating my typing on the couch, coming into lab with me in the evenings and on weekends, inspiring me, buying me those $20 tomatoes, and always giving me the last of the numerous bottles of wine we have shared. Cheers!

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

CHAPTER 1

INTRODUCTION

The Cytochrome P450s

The cytochrome P450 enzymes (P450s) are a highly conserved family of enzymes present in the cells of all prokaryotes and eukaryotes (*1*). P450s are essential to the synthesis of steroids, fatty acids, and some vitamins. P450s are also instrumental in the metabolism of both endogenous and xenobiotic substrates. It is estimated that over 50% of all therapeutics currently in use are biotransformed by the P450 enzyme family (*2*). Generally this reaction produces a more-polar, less-toxic, and more readily excretable product. In some cases, such as with the chemotherapeutic cyclophosphamide, this reaction produces a more therapeutically active compound and is thus necessary for drug function (*3*). Drugs requiring metabolic activation are referred to as prodrugs.

To date, more than 13,000 P450 genes have been identified, and 57 have shown to be present in humans as a result of the Human Genome Project (*4*). Furthermore, one quarter of the 57 human P450s play crucial roles in drug metabolism and these are depicted in Figure 1.1 (*4*). The P450s are classified by amino acid sequence similarity, such that families have 40% or greater similarity and are denoted by Arabic numbers. The families are then assigned

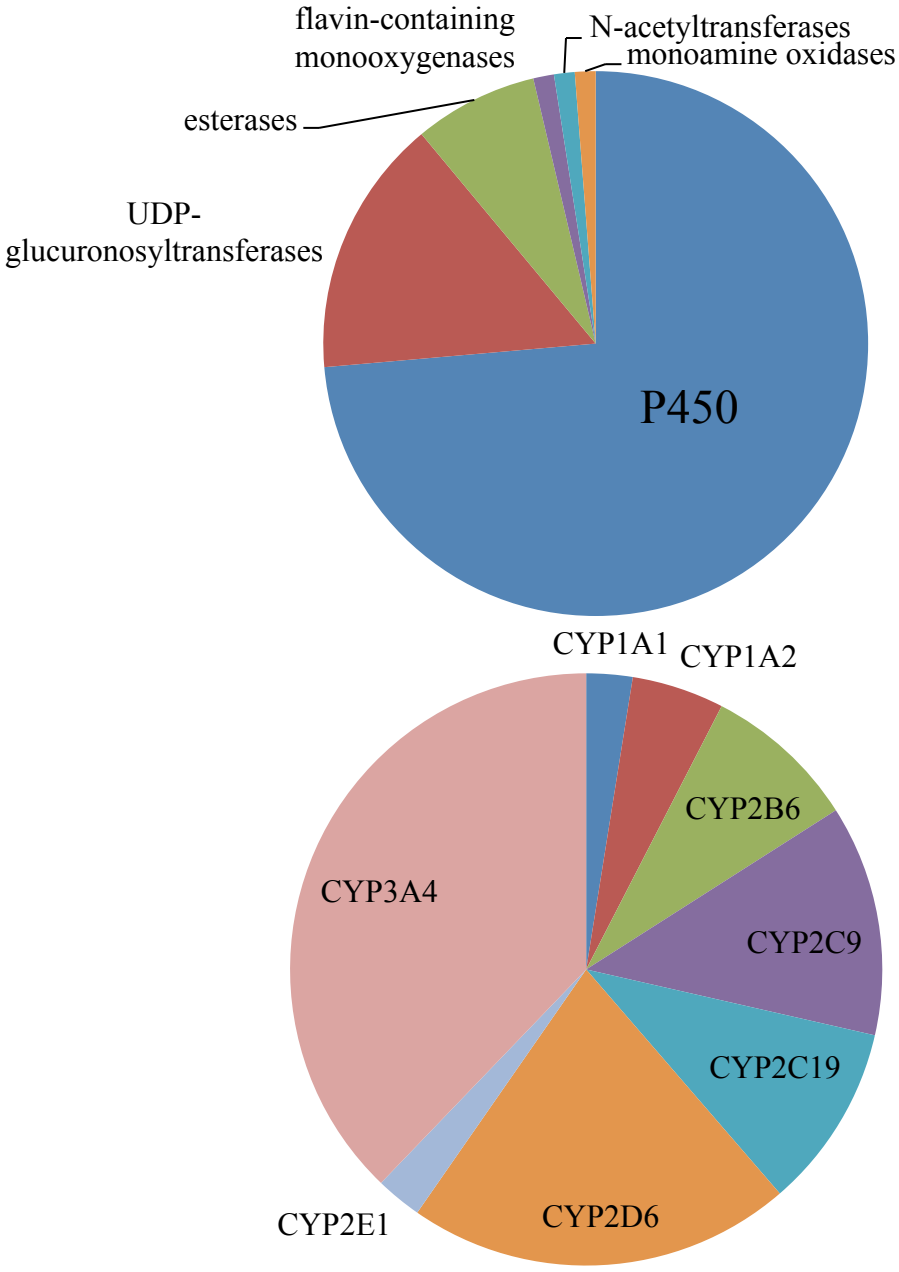


Figure 1.1 – Drug Metabolizing Enzymes. The top pie chart depicts the common enzymes involved in all drug metabolism. The bottom diagram shows the percent of P450-metabolized prescription drugs metabolized by the major human P450s. The figure is adapted from Rendic and Guengerich *(4)*.

into subfamilies, where members have 55% or greater similarity and these subfamilies are denoted by capital letters. Finally, based on the order of discovery of the P450s in the given subfamily, they are assigned a second Arabic number; for example, cytochrome P450 2B6 (*CYP2B6*). More recently, common genetic polymorphisms have been noted for most of the P450s. These common polymorphisms are denoted by a period and third Arabic number, for instance *CYP2B6.4*. The wild type allele is denoted by *CYP2B6.1* or assumed unless otherwise noted. In the case of the transcribed enzymes for these genes, they are written as CYP2B6 or CYP2B6*4 for the polymorphic variants.

The P450s are membrane-bound enzymes located in the endoplasmic reticulum or mitochondria of most tissues in mammals (*1*). The P450s have been described as "Nature's most versatile biological catalyst," because these enzymes catalyze a wide variety of reactions on a structurally diverse assortment of substrates (*5*). The most common reactions are mono-oxygenations, such as hydroxylation, epoxidation, *N*- and *O*-dealkylations and nitrogen and sulfur oxidations. Less common reactions include deformylations, dehydrogenations, and reactions that don't require oxidations, such as reductions and ring expansions (*3*). These reactions are considered to be members of the Phase I drug metabolism class of reactions in humans (*6*). The following scheme describes the overall stoichiometry for substrate hydroxylation by P450:

$$\mathbf{RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+}$$

The P450s require accessory proteins for successful catalysis. These accessory proteins, known as NADPH-cytochrome P450 reductase (reductase) and, in some cases, cytochrome b5 (b5), are located adjacent to the P450s in the endoplasmic reticulum or

mitochondrial membranes (Figure 1.2). Generally, the P450:reductase:b5 ratio in liver is 20:1:0.5 (*7*). The reductase is essential for electron transfer from the cofactor NADPH to P450 either once or twice during the P450 catalytic cycle, with the first electron always coming from the reductase. The second electron transfer can be completed either by the reductase or b5; however, b5 involvement in the cycle is known to be dependent on both the P450 and the substrate (*8*).

The detailed catalytic cycle for P450 catalyzed reactions is shown in Figure 1.3. The first step in the catalytic cycle is the binding of the substrate in the active site in close proximity to the heme group [1]. Next, the reductase transfers an electron from NADPH to the heme thereby reducing the heme [2]. One molecule of dioxygen then binds the now-ferrous heme iron [3], and a second reduction of the ferrous heme iron-dioxygen complex occurs via either reductase or b5 [4]. Two protons are taken up and heterolytic splitting of the oxygen-oxygen bond generates the putative iron-oxene species, with the concomitant release of one molecule of water [5]. Next, there is formation of the proposed substrate radical, a transient intermediate [6]. Then oxygen inserts into the substrate [7]. Finally product dissociation returns the P450 to the resting state [8]. Additionally a well-known reaction, the peroxide shunt, is shown [9]. In the shunt mechanism, hydrogen peroxide donates the oxygen atom for the substrate hydroxylation and there is no requirement for molecular oxygen or NADPH for electron donation. Alternatively, there can be a release of uncoupled products, such as superoxide (O_2^-) or hydrogen peroxide (H_2O_2), at the oxygen reduction step, which is not coupled to substrate oxygenation [10]. This results in the generation of reactive oxygen species that may damage the heme moiety of the enzyme.

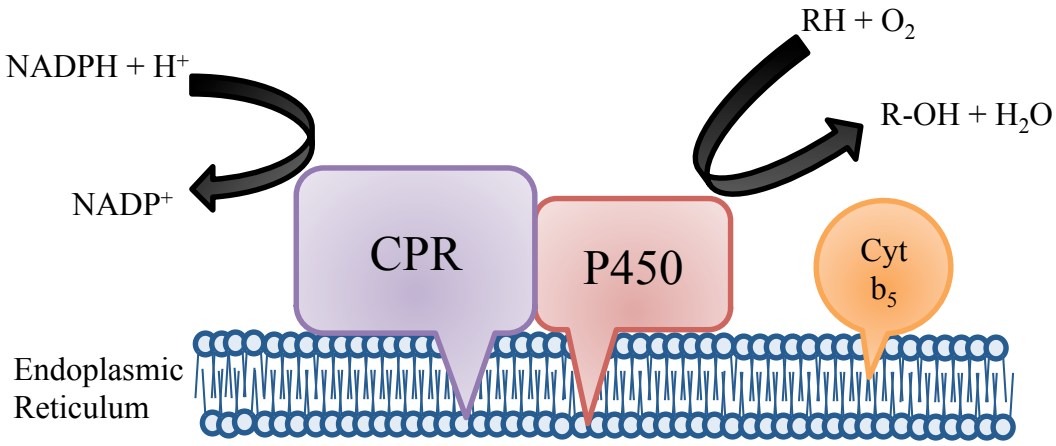


Figure 1.2 – The location of P450s and the accessory proteins and a general scheme for electron transfer. Each of the proteins, cytochrome P450 reductase (CPR), P450, or cytochrome b_5 (Cyt b_5) is embedded in the membrane of the endoplasmic reticulum via a transmembrane domain or hydrophobic helix. The proteins face the cytoplasmic side of the membrane. P450 will receive the first electron from NADPH via the CPR. Subsequently both CPR and Cyt b_5 may donate the second electron.

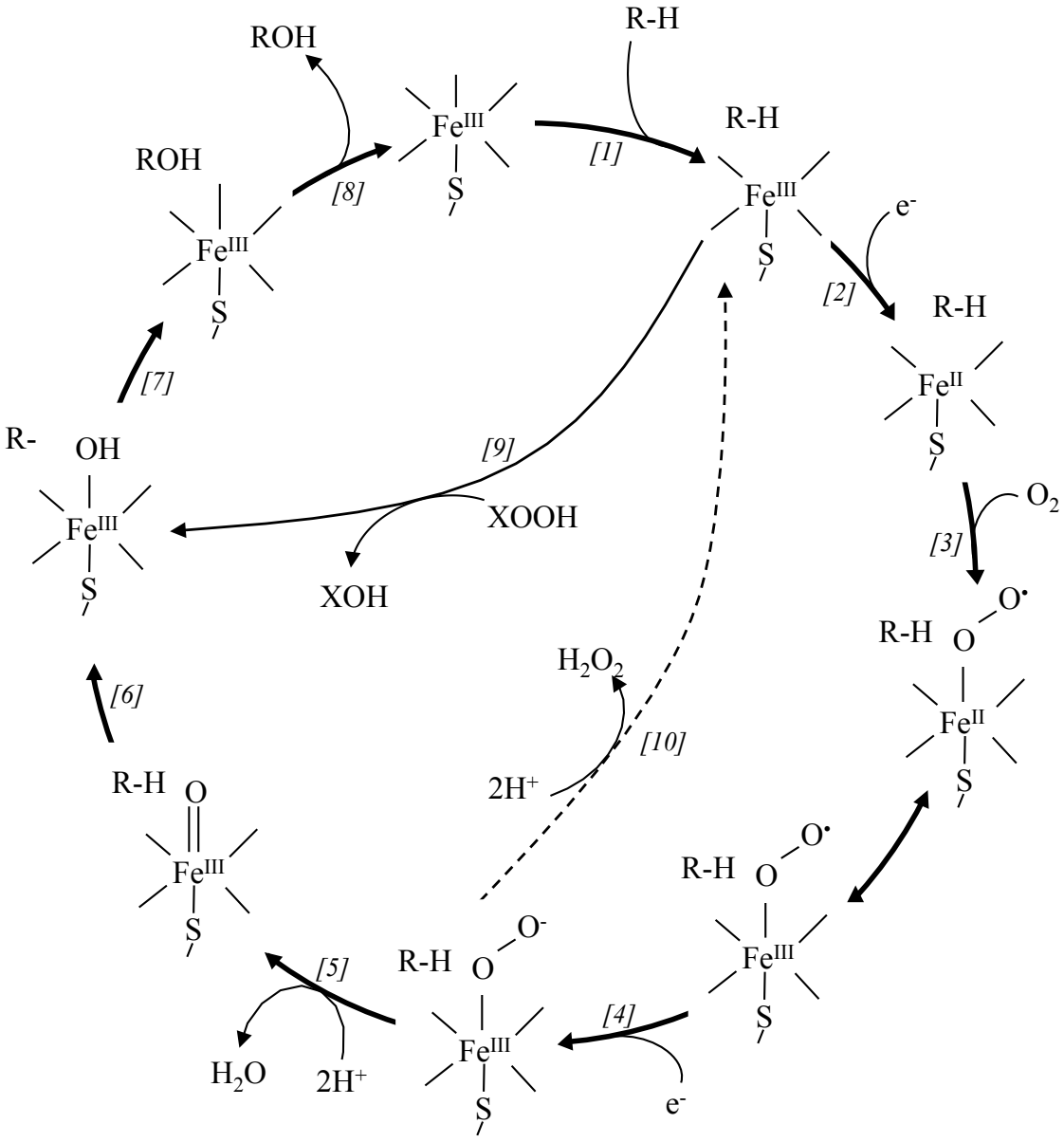


Figure 1.3 – The P450 Catalytic Cycle. See text for a detailed description of this reaction. The figure is adapted from Guengerich *(3)*.

Structure

In the early 2000's great advances were made with regards to increasing the expression levels and solubility of microsomal P450s (*9*). This enabled crystallization and eventual determination of the three dimensional structures of the P450s. To date, there are over 500 P450 X-ray structures in the Research Collaboratory for Structural Biology's Protein Data Bank (RCSB-PDB); approximately 100 of those are human P450s. P450 structures have been obtained in many conformations and for several P450s (*10*).

Generally, the soluble prokaryotic and the membrane-bound eukaryote P450s have a conserved secondary structure (as seen in Figure 1.4) (*10*). There are 12 α-helices denoted as A-L, and 4 β-sheets 1-4. Most eukaryotic P450s contain a membrane targeting sequence that anchors the P450s to the cytoplasmic surface of the endoplasmic reticulum or mitochondria. Spatially speaking, the most conserved portions of P450s are helices E, I, J, K, L, and portions of β sheet 1, these form the core of the protein and maintain the binding site for the heme prosthetic group. Substrates bind above the heme surface and are positioned close to the iron-oxo intermediate for oxidations. The outer surface of the substrate binding cavity varies depending on the P450; creating different sizes, shapes, and chemical features which allow for substrate selectivity of the enzymes (*11*).

P450 Polymorphic Variants

Genetic mutations have been described for all genes that encode the human P450s (*12*). Population frequencies for the mutations differ and are associated with ethnic groups (*12*). The functional significance of the mutation also differs and will, most likely, be

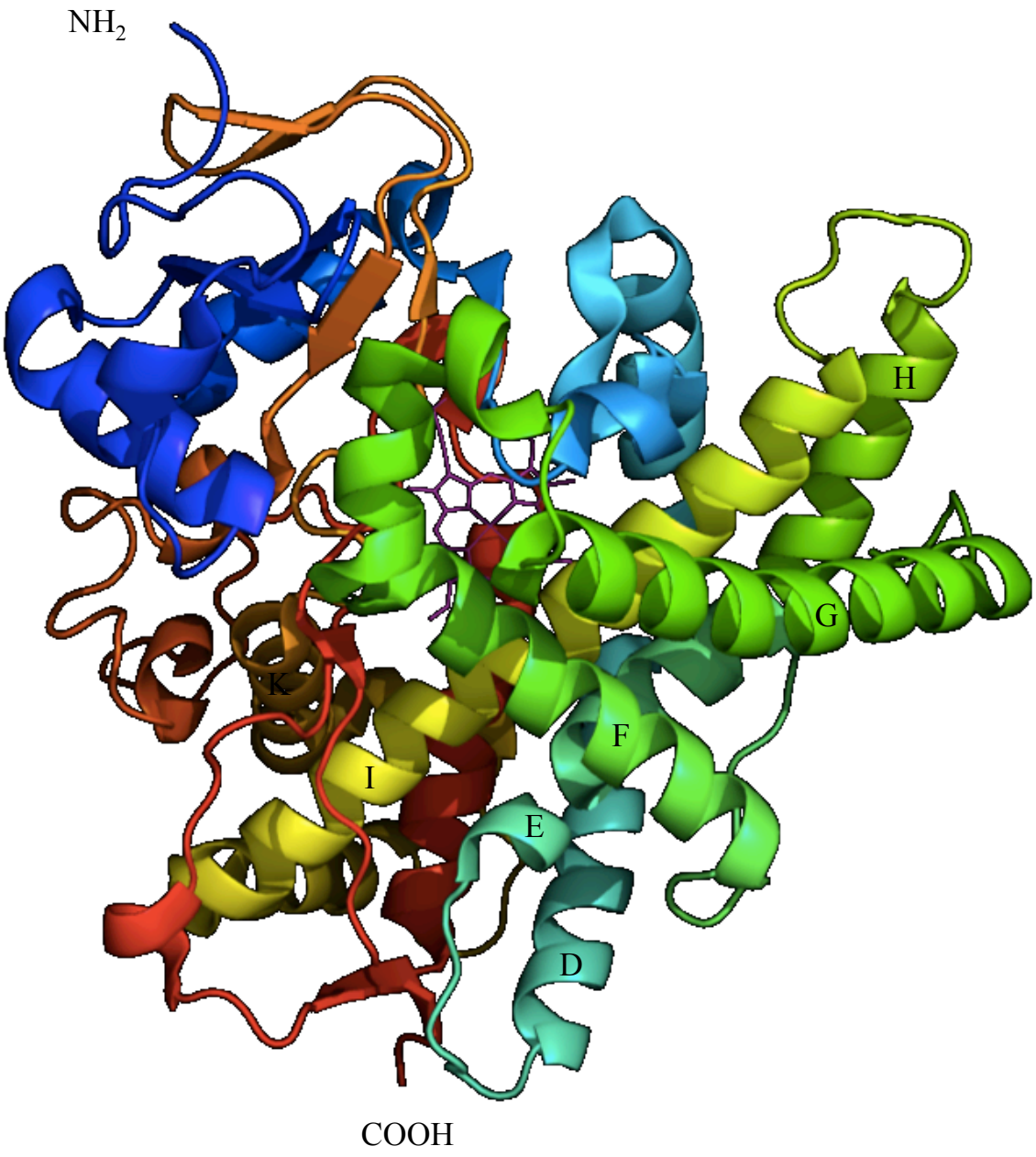


Figure 1.4 – X-Ray Crystal Structure of Human P450 CYP2B6 *(10)*. The heme moiety of the P450 is indicated by the magenta stick-structure located in the center of the structure. The helices of the P450 have been color-coordinated in reverse rainbow order, such that Helix A is in blue and Helix L is in red. The N- and C-termini have also been labeled for orientation purposes.

dependent on the substrate of interest. In general, the polymorphisms alter either the expression level and/or the catalytic efficiency of the enzyme. These characteristics of the polymorphisms can lead to significant inter-individual variability in P450 activity, which is most clearly exemplified by the dramatic variations in patient responses to drug therapy (*13*). Polymorphic forms of P450s contribute to a significant amount of adverse drug reactions (*14*). It is estimated that over-half of the drugs cited in adverse drug reaction studies are metabolized by the polymorphic phase I enzymes (86% of those enzymes being P450s, as seen in Figure 1.1) (*15*). Additionally, costs associated with treating patients who express variant alleles of the P450s are generally significantly greater than costs associated with treating patients expressing the wild type P450 (*16*). Currently, many studies are focused on correlating the genotype of the human P450s with the phenotypic outcome of drug response. It has been suggested that enhancing the knowledge of the role of polymorphic P450s in drug metabolism could result in a 10-20% improvement in clinical efficacy and reduce adverse drug reactions by 10-15% (*12*). Overall, a better understanding of the mechanism responsible for observed phenotypic variations is necessary for safer and more efficacious drug therapy regimens.

CYP2B6

Traditionally, CYP2B6 was considered an inconsequential P450 with marginal expression in the liver and a negligible role in drug metabolism. However, with the development of more sensitive and specific antibodies to the protein, as well as specific pharmacological inhibitors of the enzyme, the role of CYP2B6 became clear (*17*). It is now considered a key enzyme in drug metabolism, accounting for the metabolism of approximately 8% of the drugs administered to humans (*17*). Table 1.1 lists some of the

Table 1.1 - Common substrates, inducers, and inhibitors of P450 CYP2B6.

Substrates	Inducers	Inhibitors
cyclophosphamide	phenobarbital	thiotepa
ifosfamide	clopidogrel	efavirenz
bupropion	dexamethasone	bergamottin
methadone	cyclophosphamide	orphenadrine
efavirenz		curcumin
testosterone		
propofol		
nicotine		

common substrates for CYP2B6. Although most of the CYP2B6 substrates are converted to less-biologically active forms, the chemotherapeutic agents cyclophosphamide and ifosfamide are prodrugs and require activation by CYP2B6 to have a therapeutic effect (*18*).

As with many P450s, CYP2B6 is highly expressed in the liver, accounting for 5-10% of the total liver microsomal protein (*19*). CYP2B6 is also known to be expressed in brain (*20*), kidney (*21*), intestine (*20*), peripheral blood lymphocytes (*19*), and skin (*22*). The enzyme is subject to induction by numerous substrates, and some common inducers are listed in Table 1.1. Notably, the chemotherapeutic agent cyclophosphamide is known to induce CYP2B6 expression (*23*). CYP2B6 activities in human liver microsomes have exhibited from 20- up to 250-fold variations using S-mephenytoin as a substrate, and up to an 80-fold variation using bupropion as a substrate (*19, 24*). Variability in P450 activity can be caused by many factors including age, gender, concurrent medications, obesity and genetic polymorphisms (*12*). The genetic polymorphisms appear to have the greatest effect on the observed variability.

CYP2B6 is a highly polymorphic P450 (*25*). The Human Genome Project identified 53 distinct haplotypes, 28 characterized alleles, and numerous SNPs (*19*). A summary of the SNPs used in this thesis is in Table 1.2. Nine exonic SNPs were identified, including five amino acid variants, some of which are present in up to 30% of the Caucasian population (*25-29*). One important haplotype, *CYP2B6.4* results in a lysine to arginine point mutation at residue 262 (K262R) (*27, 28, 30*). The 262 residue is located between the G and H helices, a region that is known to be important for P450 function. Studies on bupropion metabolism have demonstrated that CYP2B6*4 produced increased amounts of the metabolite hydroxybupropion in a purified and reconstituted system (*31*). Additionally, it was

Table 1.2 - Common polymorphic variants of P450 CYP2B6 *(25- 29)*. Abbreviations are: AA – African American, CAU – Caucasian, AS – Asian (Chinese and Japanese), PNG – Papua New Guinean.

Polymorphism	Amino Acid Mutation	Mutation Location	Population Frequencies
CYP2B6*1	N.A.	N.A.	40%
CYP2B6*4	K262R	Helix H	33% in CAU 14.1% in AS
CYP2B6*5	R487C	Near N-terminus	14% in CAU 0.5% in AS
CYP2B6*6	Q172H/ K262R	Helices E & H	28% in CAU 39% in AS 32.8% in AA 62% in PNG
CYP2B6*7	Q172H/ K262R/ R487C	Helices E & H, N-terminus	3-6% in CAU
CYP2B6*8	K139E	Helix D	
CYP2B6*9	Q172H	Helix E	1% in AS

N.A. CYP2B6*1 refers to the wild type P450, therefore no mutations are present.

demonstrated that 17-α-ethynylestradiol, a mechanism-based inactivator of CYP2B6 had no effect on the catalytic activity of CYP2B6*4 (*32, 33*). These studies, as well as others on the polymorphic variants of P450s demonstrate the importance of testing the polymorphic variants for functional effects. It should also be noted that the functional effects of the variants will depend on the substrate of interest (*34*).

Cyclophosphamide

Cyclophosphamide (CPA) is one of the most successful chemotherapeutic agents ever synthesized (*18*). The chemical structure of CPA is shown in Figure 1.5. The drug also produces appreciable immune suppression and is used in low doses to treat immune disorders as well as in conditioning regimens for blood and marrow transplantation (*35*). CPA was one of the first agents rationally designed to selectively target cancer cells. The intention was to generate an inactive chemical that required activation via a phosphamidase, which is expressed at high levels in some cancer cells. Thus, the idea was to target the cancer cells with a nitrogen mustard that would be released by phosphamidase-mediated cleavage of the phosphorus-nitrogen bond (*36, 37*). Although this designed targeting mechanism failed, the drug was still highly efficacious and it was ultimately proven to rely on the P450 system for activation of the prodrug (*18*).

Studies revealed that CPA requires hydroxylation via a P450 at the 4′ carbon to become activated, as shown in Figure 1.6 (*38-40*). Once hydroxylated, 4′OH-CPA exists in equilibrium with the open ring form aldophosphamide. These unstable precursors are freely transported in the blood and diffuse readily into cells. Once in the cells, the aldophosphamide spontaneously decomposes via β-elimination to form phosphoramide

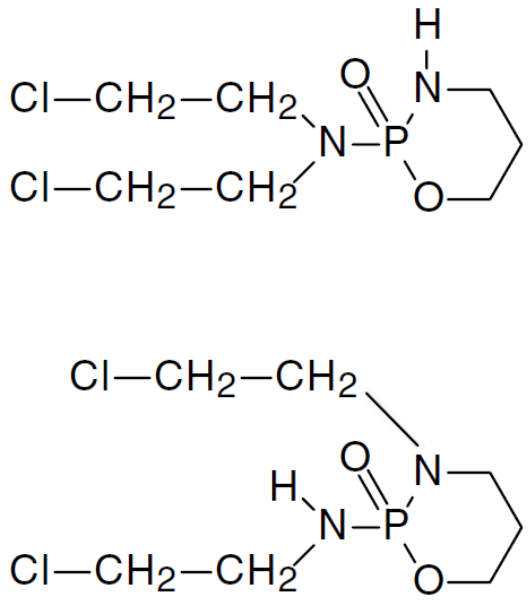


Figure 1.5 - Chemical Structures of the Oxazaphosphorines. The top structure is cyclophosphamide and the bottom is ifosfamide. Ifosfamide and cyclophosphamide differ only in the placement of one of the chloroethyl groups.

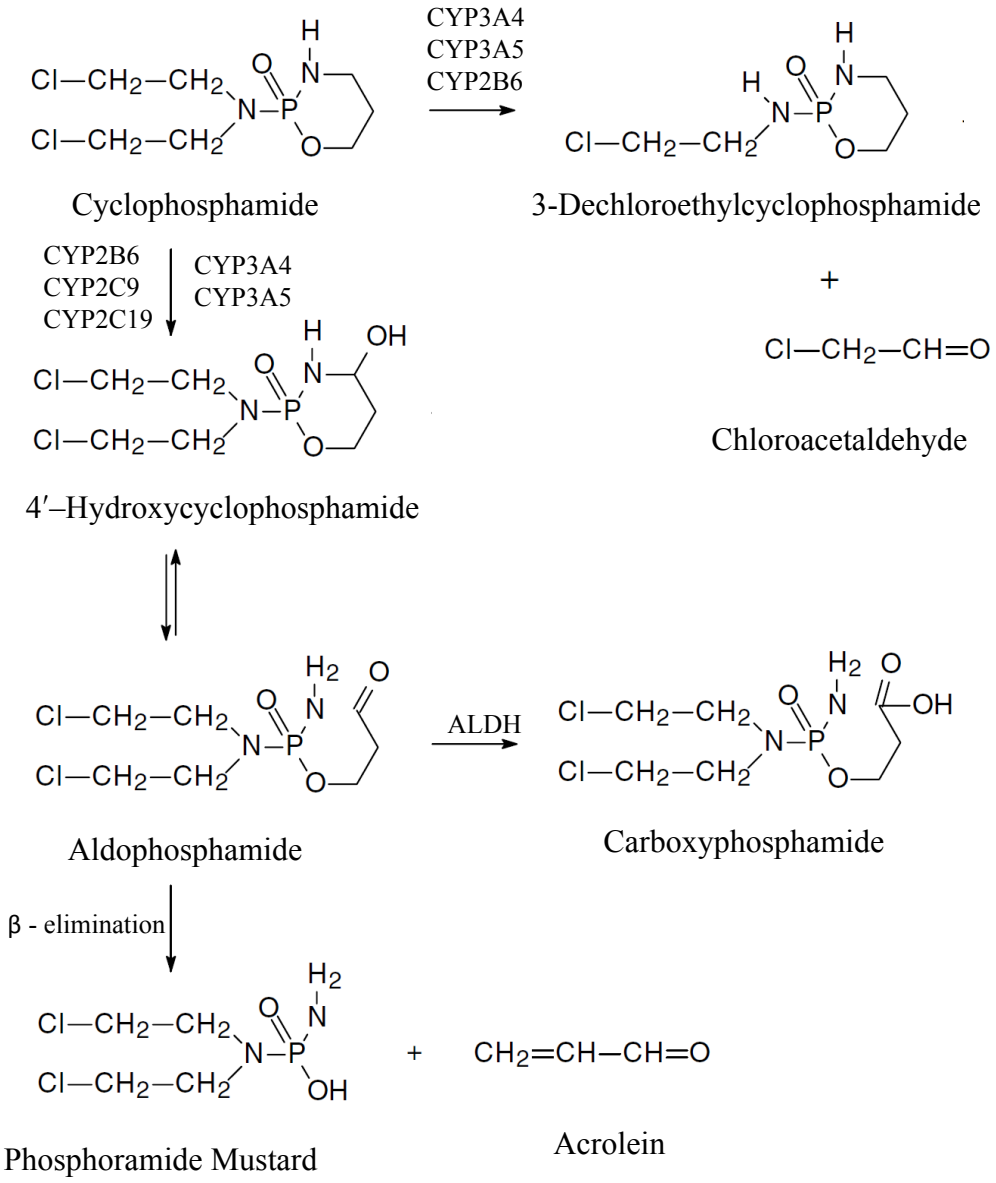


Figure 1.6 – Cyclophosphamide Metabolism. The activation pathway for CPA is indicated vertically, with the ultimate cytotoxic compound being phosphoramide mustard. The deactivation pathways are indicated horizontally. Abbreviation: ALDH – aldehyde dehydrogenase. For a more detailed description of the metabolism see the text. The figure is adapted from de Jonge *et al., (40).*

mustard and acrolein. The phosphoramide mustard then alkylates DNA base pairs, specifically on the N-7 position of guanosine bases. This modification arrests DNA replication which, when sensed by molecular mechanisms, signals for the initiation of apoptosis (*18*). Without conversion to the 4′ hydroxylated form, CPA is inactive. Many P450s have been implicated in this crucial conversion, including CYP2B6, CYP2C9, CYP2C19, and CYP3A4/5 (*41-44*). Work by Roy, *et al.* proved that CYP2B6 is the primary P450 responsible for cyclophosphamide activation (*45*). The primary pathways for CPA metabolism are shown in Figure 1.6.

CPA can also be dechloroethylated to form 2-dechloroethycyclophosphamide or 3-dechloroethycyclophosphamide, and this pathway generally accounts for 10-20% of the CPA metabolism that occurs in humans (*46*). In addition to implicating CYP2B6 as the P450 most-responsible for activating CPA, Roy *et al.* also demonstrated that CYP3A4/5 is more likely to dechloroethylate CPA (*45*). Both dechloroethylated products are known neurotoxic agents and should be monitored and CPA dosing adjusted to avoid certain toxicities associated with CPA treatment. Upon dechloroethylation, chloroacetaldehyde is also formed. This byproduct is known to cause nephrotoxicity. To avoid these toxicities, it is advantageous to promote hydroxylation of CPA at the initial metabolic step thereby converting all of the drug to the active form (*47*). Ludeman *et al.* have created several analogs of CPA in efforts to avoid dechloroethylation (*47*).

CPA is most commonly deactivated by aldehyde dehydrogenase (ALDH) after its conversion to aldophosphamide (*48*). The product, carboxyphosphamide, is the major metabolite found in urine (*49*). Cells expressing low levels of ALDH are more sensitive to the cytotoxic effects of CPA, as the conversion to and subsequent clearing of

carboxyphosphamide cannot occur. These cells include mature hematopoietic progenitors and lymphocyte subsets (*50-52*). CPA is also eliminated via glutathione S-transferases, although high levels of these enzymes *in vivo* do not confer CPA resistance (*53*).

Importantly, patient response to cyclophosphamide treatment is highly variable with regards to efficacy as well as toxicity. For example, DNA adduct accumulation in pediatric patients undergoing CPA treatment revealed great differences in the number of adducts formed in apparently similar patient cohorts (*54*). Additionally, numerous studies have demonstrated that tumor response cannot be predicted based on plasma levels of CPA (*55-59*). With regards to toxicity, the dose limiting toxic effect of CPA is cardiotoxicity. CPA-induced cardiotoxicity can be innocuous in some patients while it is fatal in others (*60*). Following high-dose CPA treatment 1-9% of patients will experience hemorrhagic necrotic perimycarditis, and 0.1% will experience fatal hemorrhagic myocarditis (*60, 61*). Additionally, 45% of patients receiving a low-dose CPA regimen will have transient cardiac toxicity (*62*). In most instances, the patient variabilities coincide with circulating levels of activated CPA (4′OH-CPA and aldophosphamide) (*63, 64*). Thus, furthering our knowledge of those factors that affect the activation and inactivation of CPA will aid in the understanding of CPA response variability. This may ultimately lead to enhanced dosing regimens for CPA such that patients will experience more of the beneficial effects of CPA and none- or less of the toxic effects.

Ifosfamide

The oxazaphosphorine ifosfamide (IFO) is also commonly used in cancer chemotherapy. Following the success of CPA, IFO was synthesized and introduced to

clinical practice in the early 1970s (*65*). Structurally, the compounds differ only in the site of one of the chloroethyl groups (Figure 1.5).

The metabolism of CPA and IFO is very similar. IFO is a prodrug that also requires hydroxylation at the 4′ carbon to become activated (Figure 1.7). Once again, CYP2B6 and CYP3A4 are the primary P450s responsible for this conversion (*44, 66*). Once converted to 4′-hydroxyifosfamide, the metabolite will exist in equilibrium with the open ring form aldoifosfamide. 4′-Hydroxyifosfamide and aldoifosfamide are considered the activated forms of the drug and they are the main circulating metabolites that will enter cells. Once inside the cell, aldoifosfamide will spontaneously decompose via β-elimination to form ifosforamide mustard and acrolein. Ifosforamide mustard is the cytotoxic agent that alkylates DNA at the N-7 positions of guanosine. Once the DNA is alkylated, DNA synthesis will be arrested and apoptosis will occur. See Figure 1.7 for the proposed pathways for the metabolism of IFO.

IFO can be dechloroethylated more readily than CPA. Approximately 25-60%, depending on the patient, of IFO is converted to either 3-dechloroethylifosfamide or 2-dechloroethylifosfamide and the byproduct chloroacetaldehyde (as compared with 10-20% of CPA) (*39, 67-69*). As with CPA, CYP3A4 is the predominant P450 responsible for this conversion (*44, 45, 70*). The dechloroethylation products, and the byproduct, chloroacetaldehyde, are known to cause neuro- and nephro-toxicities, respectively (*41*).

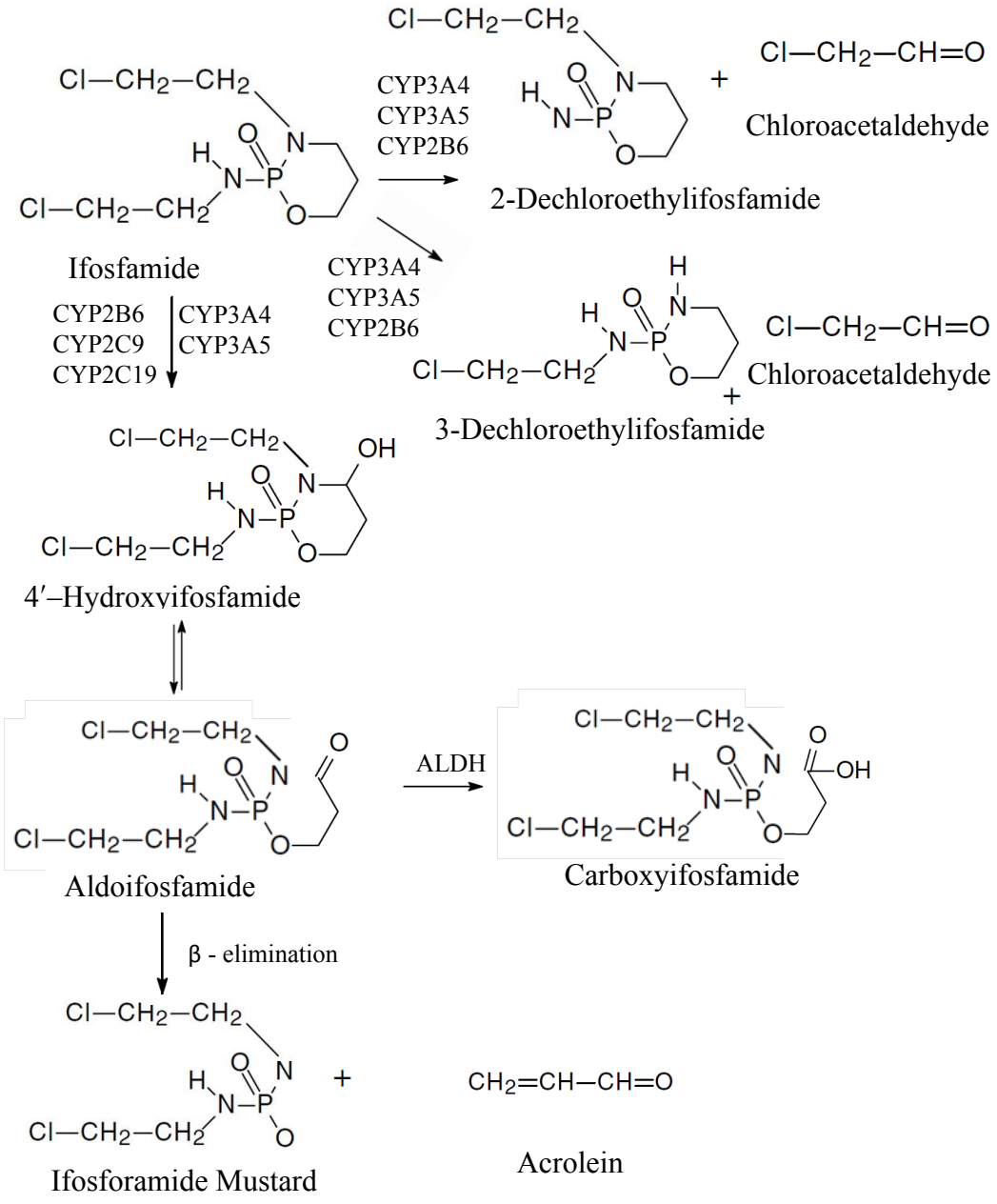


Figure 1.7 – Ifosfamide Metabolism. The activation pathway for IFO is indicated vertically, with the ultimate cytotoxic compound being ifosforamide mustard. The deactivation pathways are indicated horizontally. Abbreviation: ALDH – aldehyde dehydrogenase. For a more detailed description of the metabolism see the text. The figure is adapted from de Jonge *et al., (40).*

As compared to CPA, IFO exhibits increased hematological toxicities, bladder toxicities, nephrotoxicities, and neurotoxicities (*71*). In particular, IFO-induced toxicities are thought to be caused by the increased production of the chloroacetaldehyde byproduct (*72-74*). Moderate- to- severe nephrotoxicity is experienced in ~30% of IFO-treated patients (*75, 76*). Additionally, IFO is associated with life threatening arrhythmias, heart failure and severe encephalopathy (*35*). These adverse events limit the use of IFO, especially in pediatric patients (*35*).

IFO has proven to be more effective than CPA in a wide range of malignant diseases (*77-99*). The increased efficacy is attributed to the higher affinity of ifosforamide mustard than phosphoramide mustard for DNA. The two chloroethyl groups of ifosforamide mustard place these alkylating moieties in a different orientation and range for cross-linking DNA, resulting in increased cross-linking (*100, 101*). However, as indicated above, IFO use is limited due to the significant increases in its associated toxicities.

Scope of Thesis

Although CPA and IFO have been used clinically for over 50 years and have contributed greatly to the effective treatment of many thousands of patients with a variety of tumors, the significant patient-to-patient variation in efficacy and toxicity persists (*35*). As mentioned previously, the polymorphic variants of the P450s are known to affect the catalytic activities of the enzymes as well as the expression levels of the enzymes. Given that CPA and IFO rely on P450s for the metabolic activation as well as inactivation, it is critical to understand the effects of P450 polymorphisms on the metabolism of these two

drugs. Further improvement of our knowledge about the activation of CPA and IFO may result in improved therapeutic dosing and fewer toxicities for patients.

References

1. Guengerich, F. P. (1993) Cytochrome P450 Enzymes, *American Scientist 81*, 440-447.

2. Rendic, S. (2002) Summary of information on human CYP enzymes: human P450 metabolism data, *Drug Metab Rev 34*, 83-448.

3. Guengerich, F. P. (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity, *Chem Res Toxicol 14*, 611-650.

4. Rendic, S., and Guengerich, F. P. (2010) Update information on drug metabolism systems--2009, part II: summary of information on the effects of diseases and environmental factors on human cytochrome P450 (CYP) enzymes and transporters, *Curr Drug Metab 11*, 4-84.

5. Estabrook, R. W. (2003) A passion for P450s (rememberances of the early history of research on cytochrome P450), *Drug Metab Dispos 31*, 1461-1473.

6. Guengerich, F. P., and Rendic, S. (2010) Update information on drug metabolism systems--2009, part I, *Curr Drug Metab 11*, 1-3.

7. Backes, W. L., and Kelley, R. W. (2003) Organization of multiple cytochrome P450s with NADPH-cytochrome P450 reductase in membranes, *Pharmacol Ther 98*, 221-233.

8. Schenkman, J. B., and Jansson, I. (2003) The many roles of cytochrome b5, *Pharmacol Ther 97*, 139-152.

9. Johnson, E. F., and Stout, C. D. (2005) Structural diversity of human xenobiotic-metabolizing cytochrome P450 monooxygenases, *Biochem Biophys Res Commun 338*, 331-336.

10. Halpert, J. R. (2011) Structure and function of cytochromes P450 2B: from mechanism-based inactivators to X-ray crystal structures and back, *Drug Metab Dispos 39*, 1113-1121.

11. Poulos, T. L., Johnson, E.F. (2005) Structure of cytochrome P450 enzymes, In *Cytochrome P450: Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.), pp 87-111, Plenum Press, New York.

12. Ingelman-Sundberg, M. (2004) Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future, *Trends Pharmacol Sci 25*, 193-200.

13. van der Weide, J., and Hinrichs, J. W. (2006) The influence of cytochrome P450 pharmacogenetics on disposition of common antidepressant and antipsychotic medications, *Clin Biochem Rev 27*, 17-25.

14. Evrard, A., and Mbatchi, L. (2012) Genetic polymorphisms of drug metabolizing enzymes and transporters: the long way from bench to bedside, *Curr Top Med Chem*.

15. Phillips, K. A., Veenstra, D. L., Oren, E., Lee, J. K., and Sadee, W. (2001) Potential role of pharmacogenomics in reducing adverse drug reactions: a systematic review, *JAMA 286*, 2270-2279.

16. Spear, B. B., Heath-Chiozzi, M., and Huff, J. (2001) Clinical application of pharmacogenetics, *Trends Mol Med 7*, 201-204.

17. Wang, H., and Tompkins, L. M. (2008) CYP2B6: new insights into a historically overlooked cytochrome P450 isozyme, *Curr Drug Metab 9*, 598-610.

18. Emadi, A., Jones, R. J., and Brodsky, R. A. (2009) Cyclophosphamide and cancer: golden anniversary, *Nat Rev Clin Oncol 6*, 638-647.

19. Ekins, S., and Wrighton, S. A. (1999) The role of CYP2B6 in human xenobiotic metabolism, *Drug Metab Rev 31*, 719-754.

20. Gervot, L., Rochat, B., Gautier, J. C., Bohnenstengel, F., Kroemer, H., de Berardinis, V., Martin, H., Beaune, P., and de Waziers, I. (1999) Human CYP2B6: expression, inducibility and catalytic activities, *Pharmacogenetics 9*, 295-306.

21. Aleksa, K., Matsell, D., Krausz, K., Gelboin, H., Ito, S., and Koren, G. (2005) Cytochrome P450 3A and 2B6 in the developing kidney: implications for ifosfamide nephrotoxicity, *Pediatr Nephrol 20*, 872-885.

22. Du, L., Hoffman, S. M., and Keeney, D. S. (2004) Epidermal CYP2 family cytochromes P450, *Toxicol Appl Pharmacol 195*, 278-287.

23. Hassan, M., Svensson, U. S., Ljungman, P., Bjorkstrand, B., Olsson, H., Bielenstein, M., Abdel-Rehim, M., Nilsson, C., Johansson, M., and Karlsson, M. O. (1999) A mechanism-based pharmacokinetic-enzyme model for cyclophosphamide autoinduction in breast cancer patients, *Br J Clin Pharmacol 48*, 669-677.

24. Kirchheiner, J., Klein, C., Meineke, I., Sasse, J., Zanger, U. M., Murdter, T. E., Roots, I., and Brockmoller, J. (2003) Bupropion and 4-OH-bupropion pharmacokinetics in relation to genetic polymorphisms in CYP2B6, *Pharmacogenetics 13*, 619-626.

25. Lang, T., Klein, K., Fischer, J., Nussler, A. K., Neuhaus, P., Hofmann, U., Eichelbaum, M., Schwab, M., and Zanger, U. M. (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver, *Pharmacogenetics 11*, 399-415.

26. Davaalkham, J., Hayashida, T., Tsuchiya, K., Gatanaga, H., Nyamkhuu, D., and Oka, S. (2009) Allele and genotype frequencies of cytochrome P450 2B6 gene in a Mongolian population, *Drug Metab Dispos 37*, 1991-1993.

27. Guan, S., Huang, M., Li, X., Chen, X., Chan, E., and Zhou, S. F. (2006) Intra- and inter-ethnic differences in the allele frequencies of cytochrome P450 2B6 gene in Chinese, *Pharm Res 23*, 1983-1990.

28. Jacob, R. M., Johnstone, E. C., Neville, M. J., and Walton, R. T. (2004) Identification of CYP2B6 sequence variants by use of multiplex PCR with allele-specific genotyping, *Clin Chem 50*, 1372-1377.

29. Mehlotra, R. K., Ziats, M. N., Bockarie, M. J., and Zimmerman, P. A. (2006) Prevalence of CYP2B6 alleles in malaria-endemic populations of West Africa and Papua New Guinea, *Eur J Clin Pharmacol 62*, 267-275.

30. Zanger, U. M., Fischer, J., Klein, K., and Lang, T. (2002) Detection of single nucleotide polymorphisms in CYP2B6 gene, *Methods Enzymol 357*, 45-53.

31. Zhang, H., Sridar, C., Kenaan, C., Amunugama, H., Ballou, D. P., and Hollenberg, P. F. (2011) Polymorphic variants of cytochrome P450 2B6 (CYP2B6.4 - CYP2B6.9) exhibit altered rates of metabolism for bupropion and efavirenz: A charge-reversal mutation in the K139E variant (CYP2B6.8) impairs formation of a functional P450-reductase complex, *J Pharmacol Exp Ther*.

32. Bumpus, N. N., and Hollenberg, P. F. (2008) Investigation of the mechanisms underlying the differential effects of the K262R mutation of P450 2B6 on catalytic activity, *Mol Pharmacol 74*, 990-999.

33. Bumpus, N. N., Kent, U. M., and Hollenberg, P. F. (2006) Metabolism of efavirenz and 8-hydroxyefavirenz by P450 2B6 leads to inactivation by two distinct mechanisms, *J Pharmacol Exp Ther 318*, 345-351.

34. Watanabe, T., Sakuyama, K., Sasaki, T., Ishii, Y., Ishikawa, M., Hirasawa, N., and Hiratsuka, M. (2010) Functional characterization of 26 CYP2B6 allelic variants (CYP2B6.2-CYP2B6.28, except CYP2B6.22), *Pharmacogenet Genomics 20*, 459-462.

35. Zhang, J., Tian, Q., and Zhou, S. F. (2006) Clinical Pharmacology of Cyclophosphamide and Ifosfamide, *Curr Drug Therapy 1*, 55-84.

36. Arnold, H., Bourseaux, F., and Brock, N. (1958) Chemotherapeutic action of a cyclic nitrogen mustard phosphamide ester (B 518-ASTA) in experimental tumours of the rat, *Nature 181*, 931.

37. Friedman, O. M., Boger, E., Grubliauskas, V., and Sommer, H. (1963) Synthesis of N-Phosphorylated Derivatives of Nitrogen Mustards with Latent Cytotoxicity, *J Med Chem 6*, 50-58.

38. Colvin, M., Brundrett, R. B., Kan, M. N., Jardine, I., and Fenselau, C. (1976) Alkylating properties of phosphoramide mustard, *Cancer Res 36*, 1121-1126.

39. Sladek, N. E. (1988) Metabolism of oxazaphosphorines, *Pharmacol Ther 37*, 301-355.

40. de Jonge, M. E., Huitema, A. D., Beijnen, J. H., and Rodenhuis, S. (2006) High exposures to bioactivated cyclophosphamide are related to the occurrence of veno-occlusive disease of the liver following high-dose chemotherapy, *Br J Cancer 94*, 1226-1230.

41. Huang, Z., Roy, P., and Waxman, D. J. (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide, *Biochem Pharmacol 59*, 961-972.

42. Yu, L., and Waxman, D. J. (1996) Role of cytochrome P450 in oxazaphosphorine metabolism. Deactivation via N-dechloroethylation and activation via 4-hydroxylation catalyzed by distinct subsets of rat liver cytochromes P450, *Drug Metab Dispos 24*, 1254-1262.

43. Chen, C. S., Lin, J. T., Goss, K. A., He, Y. A., Halpert, J. R., and Waxman, D. J. (2004) Activation of the anticancer prodrugs cyclophosphamide and ifosfamide: identification of cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics, *Mol Pharmacol 65*, 1278-1285.

44. Chang, T. K., Weber, G. F., Crespi, C. L., and Waxman, D. J. (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes, *Cancer Res 53*, 5629-5637.

45. Roy, P., Yu, L. J., Crespi, C. L., and Waxman, D. J. (1999) Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles, *Drug Metab Dispos 27*, 655-666.

46. Ren, S., Yang, J. S., Kalhorn, T. F., and Slattery, J. T. (1997) Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethylcyclophosphamide in human liver microsomes, *Cancer Res 57*, 4229-4235.

47. Ludeman, S. M., D'Alessandro, M. A., Huang, R. S., Spasojevic, I., Dolan, M. E. (2012) The use of deuterium isotope effects to shift the partitioning of cyclophosphamide and ifosfamide among competing, P450-dependent, oxidative pathways, In *American Association for Cancer Research 103rd Annual Meeting*, Cancer Research, Chicago, IL.

48. Chen, T. L., Kennedy, M. J., Anderson, L. W., Kiraly, S. B., Black, K. C., Colvin, O. M., and Grochow, L. B. (1997) Nonlinear pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide/aldophosphamide in patients with metastatic breast

cancer receiving high-dose chemotherapy followed by autologous bone marrow transplantation, *Drug Metab Dispos 25*, 544-551.

49. Joqueviel, C., Martino, R., Gilard, V., Malet-Martino, M., Canal, P., and Niemeyer, U. (1998) Urinary excretion of cyclophosphamide in humans, determined by phosphorus-31 nuclear magnetic resonance spectroscopy, *Drug Metab Dispos 26*, 418-428.

50. Gordon, M. Y., Goldman, J. M., and Gordon-Smith, E. C. (1985) 4-Hydroperoxycyclophosphamide inhibits proliferation by human granulocyte-macrophage colony-forming cells (GM-CFC) but spares more primitive progenitor cells, *Leuk Res 9*, 1017-1021.

51. Ozer, H., Cowens, J. W., Colvin, M., Nussbaum-Blumenson, A., and Sheedy, D. (1982) In vitro effects of 4-hydroperoxycyclophosphamide on human immunoregulatory T subset function. I. Selective effects on lymphocyte function in T-B cell collaboration, *J Exp Med 155*, 276-290.

52. Jones, R. J., Barber, J. P., Vala, M. S., Collector, M. I., Kaufmann, S. H., Ludeman, S. M., Colvin, O. M., and Hilton, J. (1995) Assessment of aldehyde dehydrogenase in viable cells, *Blood 85*, 2742-2746.

53. Tanner, B., Hengstler, J. G., Dietrich, B., Henrich, M., Steinberg, P., Weikel, W., Meinert, R., Kaina, B., Oesch, F., and Knapstein, P. G. (1997) Glutathione, glutathione S-transferase alpha and pi, and aldehyde dehydrogenase content in relationship to drug resistance in ovarian cancer, *Gynecol Oncol 65*, 54-62.

54. Johnson, L. A., Malayappan, B., Tretyakova, N., Campbell, C., MacMillan, M. L., Wagner, J. E., and Jacobson, P. A. (2012) Formation of cyclophosphamide specific DNA adducts in hematological diseases, *Pediatr Blood Cancer 58*, 708-714.

55. Tasso, M. J., Boddy, A. V., Price, L., Wyllie, R. A., Pearson, A. D., and Idle, J. R. (1992) Pharmacokinetics and metabolism of cyclophosphamide in paediatric patients, *Cancer Chemother Pharmacol 30*, 207-211.

56. Sladek, N. E. (1972) Therapeutic efficacy of cyclophosphamide as a function of inhibition of its metabolism, *Cancer Res 32*, 1848-1854.

57. Sladek, N. E. (1972) Therapeutic efficacy of cyclophosphamide as a function of its metabolism, *Cancer Res 32*, 535-542.

58. Nieto, Y., Xu, X., Cagnoni, P. J., Matthes, S., Shpall, E. J., Bearman, S. I., Murphy, J., and Jones, R. B. (1999) Nonpredictable pharmacokinetic behavior of high-dose cyclophosphamide in combination with cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea, *Clin Cancer Res 5*, 747-751.

59. Hadidi, A. H., Coulter, C. E., and Idle, J. R. (1988) Phenotypically deficient urinary elimination of carboxyphosphamide after cyclophosphamide administration to cancer patients, *Cancer Res 48*, 5167-5171.

60. Ayash, L. J., Wright, J. E., Tretyakov, O., Gonin, R., Elias, A., Wheeler, C., Eder, J. P., Rosowsky, A., Antman, K., and Frei, E., 3rd. (1992) Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response, *J Clin Oncol 10*, 995-1000.

61. Murdych, T., and Weisdorf, D. J. (2001) Serious cardiac complications during bone marrow transplantation at the University of Minnesota, 1977-1997, *Bone Marrow Transplant 28*, 283-287.

62. Bearman, S. I., Petersen, F. B., Schor, R. A., Denney, J. D., Fisher, L. D., Appelbaum, F. R., and Buckner, C. D. (1990) Radionuclide ejection fractions in the evaluation of patients being considered for bone marrow transplantation: risk for cardiac toxicity, *Bone Marrow Transplant 5*, 173-177.

63. Anderson, L. W., Chen, T. L., Colvin, O. M., Grochow, L. B., Collins, J. M., Kennedy, M. J., and Strong, J. M. (1996) Cyclophosphamide and 4-Hydroxycyclophosphamide/aldophosphamide kinetics in patients receiving high-dose cyclophosphamide chemotherapy, *Clin Cancer Res 2*, 1481-1487.

64. Chen, T. L., Passos-Coelho, J. L., Noe, D. A., Kennedy, M. J., Black, K. C., Colvin, O. M., and Grochow, L. B. (1995) Nonlinear pharmacokinetics of cyclophosphamide in patients with metastatic breast cancer receiving high-dose chemotherapy followed by autologous bone marrow transplantation, *Cancer Res 55*, 810-816.

65. Cohen, M. H., Creaven, P. J., Tejada, F., Hansen, H. H., Muggia, F., Mittelman, A., and Selawry, O. S. (1975) Phase I clinical trial of isophosphamide (NSC-109724), *Cancer Chemother Rep 59*, 751-755.

66. Allen, L. M., and Creaven, P. J. (1975) Pharmacokinetics of ifosfamide, *Clin Pharmacol Ther 17*, 492-498.

67. Carlson, L., Goren, M. P., Bush, D. A., Griener, J. C., Quigley, R., Tkaczewski, I., Kamen, B. A., and Weitman, S. D. (1998) Toxicity, pharmacokinetics, and in vitro hemodialysis clearance of ifosfamide and metabolites in an anephric pediatric patient with Wilms' tumor, *Cancer Chemother Pharmacol 41*, 140-146.

68. Bruggemann, S. K., Kisro, J., and Wagner, T. (1997) Ifosfamide cytotoxicity on human tumor and renal cells: role of chloroacetaldehyde in comparison to 4-hydroxyifosfamide, *Cancer Res 57*, 2676-2680.

69. Borner, K., Kisro, J., Bruggemann, S. K., Hagenah, W., Peters, S. O., and Wagner, T. (2000) Metabolism of ifosfamide to chloroacetaldehyde contributes to antitumor activity in vivo, *Drug Metab Dispos 28*, 573-576.

70. Walker, D., Flinois, J. P., Monkman, S. C., Beloc, C., Boddy, A. V., Cholerton, S., Daly, A. K., Lind, M. J., Pearson, A. D., Beaune, P. H., and et al. (1994) Identification of the major human hepatic cytochrome P450 involved in activation and N-dechloroethylation of ifosfamide, *Biochem Pharmacol 47*, 1157-1163.

71. Klastersky, J. (2003) Side effects of ifosfamide, *Oncology 65 Suppl 2*, 7-10.

72. Aleksa, K., Ito, S., and Koren, G. (2004) Renal-tubule metabolism of ifosfamide to the nephrotoxic chloroacetaldehyde: pharmacokinetic modeling for estimation of intracellular levels, *J Lab Clin Med 143*, 159-162.

73. Dubourg, L., Michoudet, C., Cochat, P., and Baverel, G. (2001) Human kidney tubules detoxify chloroacetaldehyde, a presumed nephrotoxic metabolite of ifosfamide, *J Am Soc Nephrol 12*, 1615-1623.

74. Woodland, C., Ito, S., Granvil, C. P., Wainer, I. W., Klein, J., and Koren, G. (2000) Evidence of renal metabolism of ifosfamide to nephrotoxic metabolites, *Life Sci 68*, 109-117.

75. McCune, J. S., Risler, L. J., Phillips, B. R., Thummel, K. E., Blough, D., and Shen, D. D. (2005) Contribution of CYP3A5 to hepatic and renal ifosfamide N-dechloroethylation, *Drug Metab Dispos 33*, 1074-1081.

76. Loebstein, R., Atanackovic, G., Bishai, R., Wolpin, J., Khattak, S., Hashemi, G., Gobrial, M., Baruchel, S., Ito, S., and Koren, G. (1999) Risk factors for long-term outcome of ifosfamide-induced nephrotoxicity in children, *J Clin Pharmacol 39*, 454-461.

77. Pocali, B., De Simone, M., Annunziata, M., Palmieri, S., D'Amico, M. R., Copia, C., Viola, A., Mele, G., Schiavone, E. M., and Ferrara, F. (2004) Ifosfamide, epirubicin and etoposide (IEV) regimen as salvage and mobilization therapy for refractory or early relapsing patients with aggressive non-Hodgkin's lymphoma, *Leuk Lymphoma 45*, 1605-1609.

78. Ferme, C., Bastion, Y., Lepage, E., Berger, F., Brice, P., Morel, P., Gabarre, J., Nedellec, G., Reman, O., Cheron, N., and et al. (1995) The MINE regimen as intensive salvage chemotherapy for relapsed and refractory Hodgkin's disease, *Ann Oncol 6*, 543-549.

79. Mayer, J., Koristek, Z., Vasova, I., Vorlicek, J., and Vodvarka, P. (1999) Ifosfamide and etoposide-based chemotherapy as salvage and mobilizing regimens for poor prognosis lymphoma, *Bone Marrow Transplant 23*, 413-419.

80. Biagi, J. J., Herbert, K. E., Smith, C., Abdi, E., Leahy, M., Falkson, C., Wolf, M., Januszewicz, H., Seymour, J. F., Richards, K., Matthews, J. P., Dale, B., and Prince, H. M. (2005) A phase II study of dexamethasone, ifosfamide, cisplatin and etoposide (DICE) as salvage chemotherapy for patients with relapsed and refractory lymphoma, *Leuk Lymphoma 46*, 197-206.

81. Arland, M., Leuner, S., Lange, S., Bartsch, R., Kahl, C., Florschutz, A., Franke, A., and Hoffkes, H. G. (2001) Ifosamide, epirubicin and granulocyte colony-stimulating factor: a regimen for successful mobilization of peripheral blood progenitor cells in patients with multiple myeloma, *Hematol Oncol 19*, 59-66.

82. Graziano, S. L., Valone, F. H., Herndon, J. E., 2nd, Crawford, J., Richards, F., 2nd, Rege, V. B., Clamon, G., and Green, M. R. (1996) A randomized phase II study of ifosfamide/mesna/cisplatin plus G-CSF or etoposide/cisplatin plus G-CSF in advanced non-small cell lung cancer: a Cancer and Leukemia Group B study, *Lung Cancer 14*, 315-329.

83. Thomson, B., Park, J. R., Felgenhauer, J., Meshinchi, S., Holcenberg, J., Geyer, J. R., Avramis, V., Douglas, J. G., Loken, M. R., and Hawkins, D. S. (2004) Toxicity and efficacy of intensive chemotherapy for children with acute lymphoblastic leukemia (ALL) after first bone marrow or extramedullary relapse, *Pediatr Blood Cancer 43*, 571-579.

84. Donfrancesco, A., Jenkner, A., Castellano, A., Ilari, I., Milano, G. M., De Sio, L., Cozza, R., Fidani, P., Deb, G., De Laurentis, C., Inserra, A., and Dominici, C. (2004) Ifosfamide/carboplatin/etoposide (ICE) as front-line, topotecan/cyclophosphamide as second-line and oral temozolomide as third-line treatment for advanced neuroblastoma over one year of age, *Acta Paediatr Suppl 93*, 6-11.

85. Kim, D. W., Jo, Y. H., Kim, J. H., Wu, H. G., Rhee, C. S., Lee, C. H., Kim, T. Y., Heo, D. S., Bang, Y. J., and Kim, N. K. (2004) Neoadjuvant etoposide, ifosfamide, and cisplatin for the treatment of olfactory neuroblastoma, *Cancer 101*, 2257-2260.

86. Berrak, S. G., Pearson, M., Berberoglu, S., Ilhan, I. E., and Jaffe, N. (2005) High-dose ifosfamide in relapsed pediatric osteosarcoma: therapeutic effects and renal toxicity, *Pediatr Blood Cancer 44*, 215-219.

87. Buda, A., Dell'Anna, T., Signorelli, M., and Mangioni, C. (2003) Role of ifosfamide in cervical cancer: an overview, *Oncology 65 Suppl 2*, 63-66.

88. Hussain, S. A., and James, N. D. (2003) The systemic treatment of advanced and metastatic bladder cancer, *Lancet Oncol 4*, 489-497.

89. Calabro, F., and Sternberg, C. N. (2002) New drugs and new approaches for the treatment of metastatic urothelial cancer, *World J Urol 20*, 158-166.

90. Papadimitriou, C. A., Kouroussis, C., Moulopoulos, L. A., Vlahos, G., Rodolakis, A., Kiamouris, C., Diakomanolis, E., Gika, D., Michalas, S., and Dimopoulos, M. A. (2001) Ifosfamide, paclitaxel and cisplatin first-line chemotherapy in advanced, suboptimally debulked epithelial ovarian cancer, *Cancer 92*, 1856-1863.

91. Gonzalez-Martin, A., Crespo, C., Garcia-Lopez, J. L., Pedraza, M., Garrido, P., Lastra, E., and Moyano, A. (2002) Ifosfamide and vinorelbine in advanced platinum-

resistant ovarian cancer: excessive toxicity with a potentially active regimen, *Gynecol Oncol 84*, 368-373.

92. Kosmas, C., Tsavaris, N., Malamos, N., Stavroyianni, N., Gregoriou, A., Rokana, S., and Polyzos, A. (2003) Phase I-II study of docetaxel and ifosfamide combination in patients with anthracycline pretreated advanced breast cancer, *Br J Cancer 88*, 1168-1174.

93. Kosmas, C., Tsavaris, N., Mylonakis, N., Tsakonas, G., Gassiamis, A., Skopelitis, H., Polyzos, A., Malamos, N., and Karabelis, A. (2007) Docetaxel-ifosfamide combination in patients with advanced breast cancer failing prior anthracycline-based regimens: results of a phase I-II study, *J Chemother 19*, 322-331.

94. Sorio, R., Lombardi, D., Spazzapan, S., La Mura, N., Tabaro, G., and Veronesi, A. (2003) Ifosfamide in advanced/disseminated breast cancer, *Oncology 65 Suppl 2*, 55-58.

95. Seo, J. H., Whang, Y. M., Kim, B. S., Choi, C. W., Shin, S. W., Kim, Y. H., Kim, J. S., and Goo, B. H. (2002) A Phase II study of paclitaxel by 24-hour infusion and ifosfamide in anthracycline-resistant metastatic breast carcinoma, *Cancer 94*, 1925-1930.

96. Masters, G. A., Hoffman, P. C., Drinkard, L. C., Samuels, B. L., Golomb, H. M., and Vokes, E. E. (1998) A review of ifosfamide and vinorelbine in advanced non-small cell carcinoma of the lung, *Semin Oncol 25*, 8-14.

97. Lorigan, P., Woll, P. J., O'Brien, M. E., Ashcroft, L. F., Sampson, M. R., and Thatcher, N. (2005) Randomized phase III trial of dose-dense chemotherapy supported by whole-blood hematopoietic progenitors in better-prognosis small-cell lung cancer, *J Natl Cancer Inst 97*, 666-674.

98. Bourgeois, H., Billiart, I., Chabrun, V., Chieze, S., Lemerre, D., Germain, T., Ferrand, V., Meurice, J. C., Daban, A., and Tourani, J. M. (2004) Phase I study with dose escalation of gemcitabine and cisplatin in combination with ifosfamide (GIP) in patients with non-small-cell lung carcinoma, *Am J Clin Oncol 27*, 89-95.

99. Smith, I. E., Perren, T. J., Ashley, S. A., Woodiwiss, J., Forgeson, G. V., Yarnold, J. R., and Ford, H. T. (1990) Carboplatin, etoposide, and ifosfamide as intensive chemotherapy for small-cell lung cancer, *J Clin Oncol 8*, 899-905.

100. Springer, J. B., Colvin, M. E., Colvin, O. M., and Ludeman, S. M. (1998) Isophosphoramide Mustard and Its Mechanism of Bisalkylation, *J Org Chem 63*, 7218-7222.

101. Boal, J. H., Williamson, M., Boyd, V. L., Ludeman, S. M., and Egan, W. (1989) 31P NMR studies of the kinetics of bisalkylation by isophosphoramide mustard: comparisons with phosphoramide mustard, *J Med Chem 32*, 1768-1773.

CHAPTER 2

METABOLIC ACTIVATION AND INACTIVATION OF CYCLOPHOSPHAMIDE BY THE HUMAN CYTOCHROME P450S AND SOME COMMONLY OCCURRING CYTOCHROME P450 POLYMORPHIC VARIANTS

Introduction

CPA is commonly used in the treatment of breast and other cancers. In spite of its widespread use, there is still much to elucidate with regards to its metabolism. CPA is converted metabolically to both activated and inactivated forms by the P450s (*1*). The balance of these two pathways is critical for the efficacy and the associated-toxicities of CPA. Significant variations in exposure to the metabolites of either or both pathways may account for the large interpatient discrepancies often observed with CPA treatment.

CPA is a prodrug that is bioactivated by hydroxylation at the 4′ carbon. The activation is catalyzed by a number of human P450s including CYP2B6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 (*2*-*6*). CYP2B6 is the primary P450 responsible for metabolic activation in HLM (*7*). Following hydroxylation on the 4′ carbon, the product undergoes a series of spontaneous events to produce phosphoramide mustard, which is the active form of the drug. The mechanism for CPA activation is shown in Figure 1.6.

CPA can also be inactivated via P450-mediated dechloroethylation of either or both of the chloroethyl groups. Of the human P450s, CYP3A4/5 are the most likely to catalyze this reaction (*8*). This pathway accounts for ~20% of CPA metabolism that occurs in humans (*1, 4, 9*). Dechloroethylation produces chloroacetaldehyde (CAA) and either 2-dechloroethycyclophosphamide or 3-dechloroethycyclophosphamide (Figure 1.6). The dechloroethylated products are potent neurotoxic agents and CAA causes nephrotoxicity, which can be dose limiting in some patients (*3, 10-12*). To enhance CPA efficacy and minimize these toxicities, it is advantageous to avoid dechloroethylation and promote hydroxylation of CPA.

A hallmark of CPA treatment is variable interpatient response. For example, pediatric patients undergoing CPA treatment exhibit differing amounts of DNA adduct accumulation, and numerous studies have demonstrated that tumor response cannot be predicted based only on CPA plasma levels (*13-18*). Additionally, cardiotoxicity is not observed in some patients, yet is fatal in others (*19*). Following high-dose CPA treatment, 1 to 9% of patients will experience hemorrhagic necrotic perimycarditis and 0.1% will experience fatal hemorrhagic myocarditis (*19, 20*). Additionally, 45% of patients receiving low-dose CPA will experience transient cardiac toxicity (*21*). In most cases the incidence of toxicities in patients correlates with the circulating levels of activated CPA metabolites (*22, 23*). The elucidation of those factors affecting CPA metabolism may aid our understanding of the variability in CPA response and have the potential to enhance CPA dosing regimens such that patients will experience increases in the beneficial effects of CPA and the toxic effects will be reduced.

The P450 polymorphic variants have been shown to exhibit different metabolic properties than the wild type enzymes (*24-29*). Thus, interpatient variabilities in CPA response may be due in part to altered CPA metabolism by the P450 polymorphic variants. Carriers of the *CYP2B6.4* or *CYP2B6.6* alleles have been found to be more sensitive to CPA-induced toxicities (*30-32*). Also, carriers of the *CYP2B6.5* allele exhibit poorer responses to CPA treatment (*33, 34*). Using the purified reconstituted system, Raccor *et al.* observed differences in CPA activation by CYP2B6 polymorphic variants, but concluded that overall the variants did not exhibit significantly altered activation kinetics (*35*). However, they did not investigate the inactivation pathway for CPA. This is important since the P450 polymorphic variants may exhibit altered rates for CPA inactivation that could result in increased circulating levels of the toxic metabolites.

For these studies, we utilized the reconstituted system to determine the relative efficiencies for the activation and inactivation of CPA by those P450s thought to be important in its metabolism. We also investigated the differences in CPA activation and inactivation by the polymorphic variants; CYP2B6 (CYP2B6*4-*9) and CYP2C9 (CYP2C9*2, *3). Finally, we investigated the effect of P450 inhibition on the balance of the pathways in HLM. Our ultimate goal was to examine the possibility of modulating CPA metabolism using clinically relevant FDA-approved drugs known to be specific inhibitors of the primary P450s involved in CPA metabolism

Experimental Procedures

Materials. NADPH, catalase, CPA, L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3- phosphocholine, L-α-phosphatidylserine, acrolein, CAA, 3-aminophenol, and adenosine

were all purchased from Sigma Aldrich (St. Louis, MO). Pooled HLM were purchased from BD Biosciences (San Jose, CA). All other reagents were reagent grade chemicals purchased from standard sources.

cDNA Expression and Purification of Human P450s, P450 Polymorphic Variants, and Rat Cytochrome P450 Reductase. The polymorphic variants of CYP2B6 (CYP2B6*4 – CYP2B6*9) were constructed as previously described (*24*). All wild type and polymorphic variants were overexpressed individually in *E. coli* C41 (DE3) cells and purified as reported previously (*24*). All P450s were expressed as N-terminally truncated forms to increase the expression yields (*36, 37*). Rat CPR was expressed and purified as previously described (*38*). CYP3A5 was a gift from Dr. James Halpert (UCSD), and CYP2C9*2 and CYP2C9*3 were gifts from Dr. Tim Tracy (University of Kentucky) (*39-41*).

CPA Metabolism Assay. Metabolism of CPA by the P450s was carried out in a reconstituted system. The system consisted of P450 and CPR incubated at a 1:2 molar ratio for 30 min at 20°C. Dilauroylphosphatidylcholine was absent from the reconstituted systems containing CYP2B6, CYP2C9, and CYP2C19 because it was not required for catalysis. CYP3A4 and CYP3A5 require a mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine (1:1:1) for function. To accommodate this, 60 μg of this lipid mixture was added to the reconstitution mixture following mixing of the proteins and the system was incubated for 45 min at 4°C prior to use. The final reaction mixtures contained 0.5 μg of P450, 1 μg of CPR, 30 mM potassium phosphate (pH 7.4), 15 units of catalase, and 0-10 mM CPA in a total volume of 95 μL. The

reaction mixtures were incubated at 37°C for 4 min then a 5 μL aliquot of NADPH (final 1 mM) was added to initiate the reaction. After a 15 min incubation at 37°C, the reactions were stopped by the addition of 40 μL of ice-cold 5.5% zinc sulfate, 40 μL of ice-cold saturated barium hydroxide, and 20 μL of ice-cold 0.01M HCl (*42*). The samples were centrifuged for 10 min at 13,200 rpm and kept on ice prior to analysis for acrolein and CAA accumulation as described below. The reaction conditions were optimized such that product formation was within the linear range for time and protein concentration of the reaction.

CPA Metabolism by Human Liver Microsomes. CPA metabolism by HLM was measured as described above, with the substitution of 0.2 mg/mL of HLM for the reconstituted protein mixture. Additionally, the reaction mixture was incubated for 30 min at 37°C in order to increase product formation. To inhibit specific P450s, 5 μM ketoconazole (CYP3A4/5 inhibition) or 5 μM clopidogrel (CYP2B6 inhibition) was added to the reaction mixtures prior to initiating the reaction with NADPH.

Quantification of Acrolein Formation. In order to measure the formation of acrolein, 60 μL of the reaction mixture was incubated with 40 μL of fluorescence reagent (30 mg 3-aminophenol and 30 mg hydroxylamine HCl dissolved in 5 mL 1N HCl) for 25 min at 90°C to create the fluorescent product 7-hydroxyquinoline (*42*). Using a Shimadzu HPLC system, the samples were separated on an Agilent C_{18} column. The column was eluted isocratically with 20% methanol in 0.1% phosphoric acid at 0.5 mL/min. Elution of 7-hydroxyquinoline occurred at 3.2 min as monitored fluorometrically at 350 nm (excitation) and 515 nm (emission). The calibration curves for acrolein quantification were generated by incubation

of authentic acrolein in reaction mixtures containing no substrate or NADPH. No differences were observed between the two standard preparations. The standard curve was linear for the range of values analyzed (0-100 μM acrolein).

Quantification of Chloroacetaldehyde Formation. To determine the amount of CAA formed, 60 μL of the reaction mixture was incubated with 10 μL of 100 mM adenosine in 0.25 N HCl and 10 μL of 2 M sodium acetate (pH 4.5) for 2.5 hours at 80°C. This produced the fluorescent product, 1-*N*-ethenoadenosine (*42*). The derivatized sample was analyzed using an Agilent C_{18} column with a mobile phase of 20% methanol in water. The product eluted at 13.2 min and was monitored for fluorescence by excitation at 270 nm and emission at 411 nm. The calibration curves for quantification of CAA were generated by incubating authentic standards of CAA in reaction mixtures containing no substrate or NADPH. The standard curve was linear over the range of 0-150 μM.

Data Analysis. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc.). The parameters for enzyme kinetics were determined using non-linear regression analysis, specifically Michaelis-Menten analysis in GraphPad Prism. Statistical significance was determined using an unpaired t-test in GraphPad Prism (P value ≤ 0.05). Data were obtained in triplicate for each point, except where otherwise noted.

Results

Metabolic Activation of Cyclophosphamide by the Human Wild Type P450s. The ability of individual P450s to metabolize CPA via the activation pathway was assessed using the

reconstituted P450 system. Acrolein was used as the marker to assess activation of CPA since it is produced in a 1:1 ratio with respect to the initial 4′ hydroxylated product (*1*). P450s CYP2B6 and CYP2C19 were the only P450s tested to show substantial activity in this pathway (Figure 2.1, A). Wild type CYP2B6 exhibited a K_m of 0.6 mM and a V_{max} of 22.8 pmol/min/pmol P450. The efficiency of the enzyme for this reaction was calculated as V_{max}/K_m, and was 38 $min^{-1}mM^{-1}$ for CYP2B6. CYP2C19 exhibited a lower V_{max} value and a higher K_m, equating to a lesser efficiency (3.2 $min^{-1}mM^{-1}$) for the conversion of CPA to acrolein when compared with CYP2B6. Although CYP2C9, CYP3A4, and CYP3A5 have all been reported to catalyze this pathway, they showed very little activity in this study (*8*). All of the experimentally determined K_m and V_{max} values are shown in Table 2.1. As can be seen, the efficiencies of the P450s were: CYP2B6>CYP2C19>CYP3A4≥CYP3A5>CYP2C9.

Inactivation of Cyclophosphamide by the Human Wild Type P450s. The wild type P450s were assessed for their ability to metabolize CPA to CAA as a measure of CPA inactivation. CAA was used to quantitate metabolism via this pathway since it is formed in equimolar amounts with the pharmacologically inactive dechloroethylcyclophosphamide (*43*). Only CYP3A4 was capable of significantly catalyzing CAA formation (Figure 2.1, B). The K_m and V_{max} values for this reaction were 0.04 mM and 0.3 pmol/min/pmol P450, respectively. Surprisingly, the closely related CYP3A5 did not catalyze CAA formation. Additionally, none of the other P450s (CYP2B6, CYP2C9, and CYP2C19) that we investigated produced measureable amounts of CAA. The data are summarized in Table 2.1.

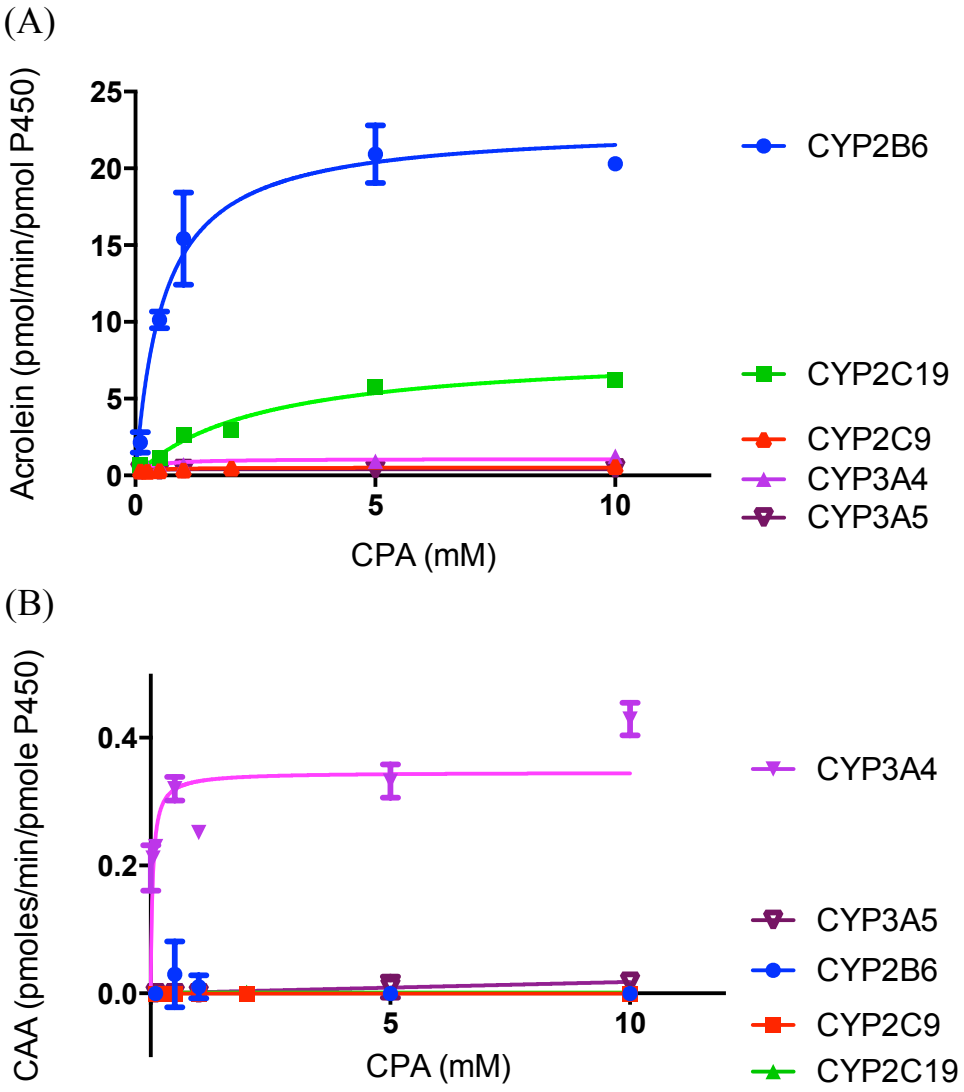


Figure 2.1 - The steady-state activities of the human wild type P450s for the metabolism of CPA. CPA (0.1 – 10 mM) was incubated in the reconstituted system containing 1 μM of each of the P450s for 15 min at 37°C. Half of each sample was analyzed for the metabolite acrolein to determine the activation kinetics of CPA (A). The other half of each sample was used to measure CAA to determine the inactivation kinetics of CPA by each of the P450s (B). Shown are the nonlinear fits determined using the Michaelis-Menten equation with Prism 6 (GraphPad Software, Inc.) for each of the P450s.

Table 2.1 - Determination of the K_m and V_{max} values for the activation and inactivation of CPA by the human wild type P450s. The K_m and V_{max} values were obtained by fitting the data shown in Figure 2 to the Michaelis-Menten equation as described under *Materials and Methods*. The reported values are averages that were determined from experiments performed in triplicate.

P450	Activation of CPA (Metabolite, Acrolein)			Inactivation of CPA (Metabolite, CAA)		
	V_{max}[a]	K_m[a]	V_{max}/K_m	V_{max}[a]	K_m[a]	V_{max}/K_m
	pmol/min/pmol P450	*mM*	$min^{-1}mM^{-1}$	*pmol/min/pmol P450*	*mM*	$min^{-1}mM^{-1}$
CYP2B6	22.8 ± 1.5	0.6 ± 0.1	38	no CAA observed		0
CYP2C9	0.2 ± 0.2	0.5 ± 0.1	0.4	no CAA observed		0
CYP2C19	8.2 ± 0.7	2.6 ± 0.6	3.2	no CAA observed		0
CYP3A4	0.3 ± 0.1	0.3 ± 0.1	1	0.3 ± 0.04	0.04 ± 0.03	7.5
CYP3A5	0.4 ± 0.04	0.04 ± 0.04	1	no CAA observed		0

[a] Mean ± S.E.M

The Metabolic Activation of Cyclophosphamide by the Polymorphic Variants of CYP2B6 and CYP2C9. In order to ascertain potential differences in the activation of CPA by some commonly expressed polymorphic variants of the human P450s, CYP2B6*4-CYP2B6*9, CYP2C9*2, and CYP2C9*3 were analyzed for the ability to catalyze acrolein formation. Each of the polymorphisms were assayed in the presence of 0.1 and 1 mM CPA, both of which are within the range of circulating plasma levels of CPA observed in patients (0.1-0.7 mM) (*19, 23*). Only the 1 mM CPA data are shown, although the trends were the same in both data sets. The averages (calculated from triplicate samples) of the total amount of acrolein formed for each polymorphism and the percentage of acrolein produced for each polymorphism as compared to the total acrolein produced by the wild type are presented (Table 2.2). CYP2B6*1 produced the most acrolein. CYP2B6*7 and CYP2B6*9 produced slightly less than half the acrolein produced by the wild type CYP2B6 at 1 mM CPA. All of the other variants tested produced significantly less acrolein than the wild type CYP2B6. The polymorphic variants of CYP2C9 did not exhibit any significant activity for the activation of CPA.

Inactivation of Cyclophosphamide by the Polymorphic Variants of CYP2B6 and CYP2C9. The amount of the inactive metabolite produced by the variants was also measured following incubation with 0.1 and 1 mM CPA. Only the data for 1 mM CPA are shown (Table 2.2). None of the polymorphic forms of CYP2B6 or CYP2C9 produced significant amounts of CAA.

Table 2.2 - Metabolism of CPA by commonly expressed polymorphic variants of CYP2B6 and CYP2C9. That data are expressed as metabolite formation in pmol/min/pmol P450, and percent of metabolite produced compared with the amount of metabolite produced by the wild type P450. The data were determined in triplicate, with the exception of CYP2B6*5 which was performed in duplicate. Statistical significance was determined using the unpaired t-test in Prism 6 (GraphPad Inc.).

P450	Activation of CPA (Metabolite, Acrolein)		Inactivation of CPA (Metabolite, CAA)	
	Acrolein Formed[a]	Percentage of Wild Type Acrolein Formed	CAA Formed[a]	Percentage of Wild Type CAA Formed
	pmol/min/pmol P450	*%*	*pmol/min/pmol P450*	*%*
CYP2B6*1	15.3 ± 3.0	100	0.01 ± 0.01	100
CYP2B6*4	0.51 ± 0.1*	3.3	0.04 ± 0.02	400
CYP2B6*5	1.8 ± 0.2*	11.7	0.0 ± 0.0	0
CYP2B6*6	0.3 ± 0.1*	2.0	0.0 ± 0.0	0
CYP2B6*7	7.1 ± 0.8	46.4	0.03 ± 0.03	300
CYP2B6*8	0.0 ± 0.0*	0.0	0.0 ± 0.0	0
CYP2B6*9	6.0 ± 0.3	39.2	0.02 ± 0.02	200
CYP2C9*1	0.1 ± 0.1	100	0.0 ± 0.0	N.A.
CYP2C9*2	0.06 ± 0.03	60.0	0.0 ± 0.0	N.A.
CYP2C9*3	0.0 ± 0.01	0.0	0.0 ± 0.0	N.A.

* $P < 0.05$

[a] Mean ± S.E.M.

N.A. no CAA was detected with wild type CYP2C9, therefore the percentage of wild type metabolite formed could not be calculated.

Cyclophosphamide Metabolism by Human Liver Microsomes with Inhibition of Specific P450s. Using pooled HLM, the activation and inactivation of CPA were measured at concentrations of 0.1, 1.0, and 10.0 mM CPA (Figure 2.2). These concentrations were chosen to reflect the range of CPA concentrations observed in the plasma of human patients (0.1-0.7 mM), as well as the potentially more concentrated levels of the drug that may occur in tissues and cells (*19, 23*). In the presence of the vehicle control, dose dependent increases in the formation of both acrolein and CAA were observed, as expected (Figure 2.2). Acrolein production was between 5 to 30-fold higher than CAA production for each of the CPA concentrations tested (Table 2.3).

Ketoconazole (5 μM) was added to HLM to inhibit CYP3A4 and the two primary routes for the metabolism of CPA were measured. Inhibition of CYP3A4 resulted in a significant decrease in the production of acrolein for all of the CPA concentrations tested (55.6-62.2% inhibition) (Figure 2.2, A and Table 2.3). In the presence of ketoconazole, the inactivation of CPA was inhibited significantly and the production of the toxic metabolite CAA was eliminated or nearly-eliminated (67.7-100.0% inhibition) at all concentrations (Figure 2.2, B and Table 2.3).

Clopidogrel (5 μM) was used to inhibit the CYP2B6 activity in HLM. Clopidogrel treatment resulted in a significant reduction of the activation of CPA. At 10 mM CPA, acrolein production was inhibited 80% (Table 2.3). CYP2B6 inhibition did not affect CAA production at 0.1 or 1 mM CPA (Figure 2.2, B). Surprisingly at 10 mM CPA, the production of CAA was not inhibited, but actually increased more than 2-fold (Table 2.3).

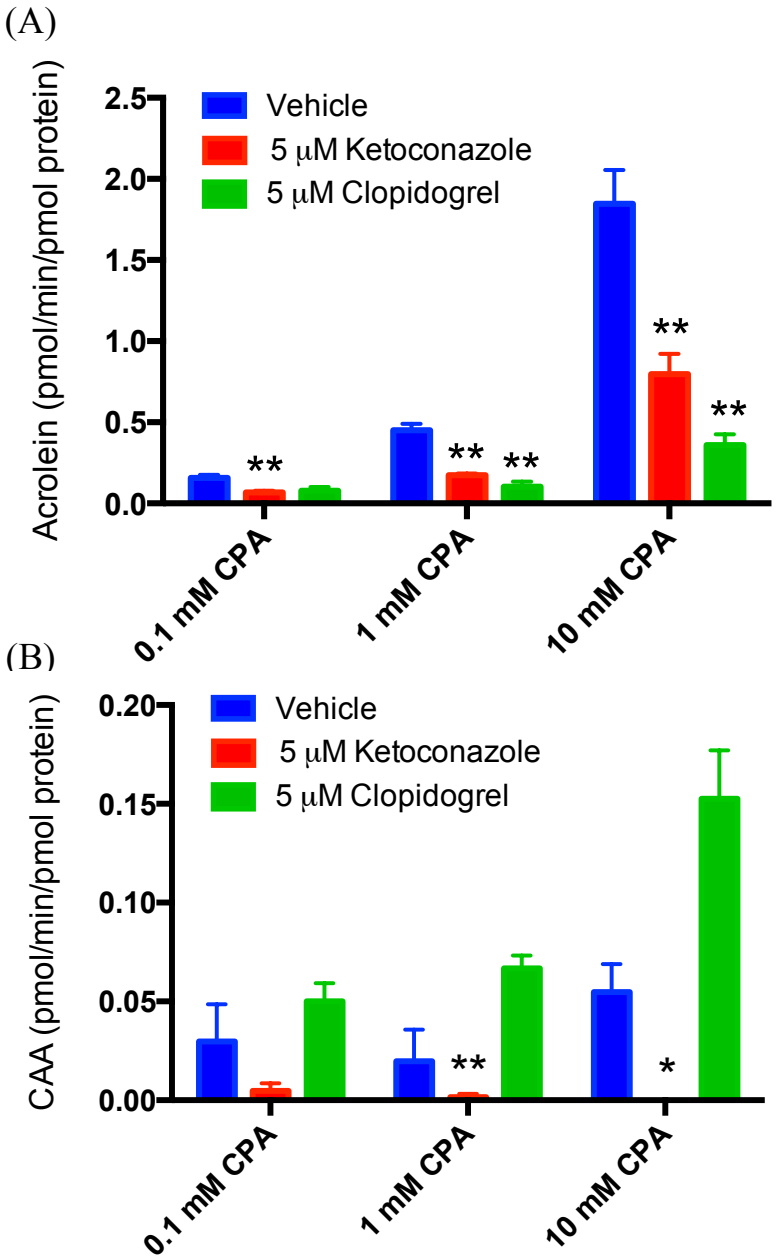


Figure 2.2 - Metabolism of CPA in HLM with P450 inhibition. The production of acrolein (A) or the inactive metabolite CAA (B) was determined at 0.1, 1.0, and 10.0 mM CPA in the presence of vehicle, 5 μM ketoconazole, or 5 μM clopidogrel. The experiments were performed in duplicate, and statistical significance was determined using an unpaired t-test in Prism 6 (GraphPad Software, Inc.), * $P < 0.05$; ** $P < 0.01$.

Table 2.3 - Summary of CPA metabolism by HLM in the presence of P450 inhibitors. The total amounts of acrolein or CAA produced by each of the experimental conditions are shown. Additionally, the percent inhibition for each of the metabolites in the ketoconazole-treated and clopidogrel-treated samples has been calculated. Note that the formation of CAA in the clopidogrel-treated samples increased. Statistical significance was determined using the unpaired t-test (P value = 0.05) in Prism 6 (GraphPad Inc.).

	Vehicle Treated		5 μM Ketoconazole Treated				5 μM Clopidogrel Treated			
	Acrolein Formed[a]	CAA Formed[a]	Acrolein Formed[a]	Inhib	CAA Formed[a]	Inhib	Acrolein Formed[a]	Inhib	CAA Formed[a]	Inhib[b]
CPA (mM)	*pmol/min/pmol P450*	*pmol/min/pmol P450*	*pmol/min/pmol P450*	*%*	*pmol/min/pmol P450*	*%*	*pmol/min/pmol P450*	*%*	*pmol/min/pmol P450*	*%*
0.1	0.16 ± 0.02	0.03 ± 0.02	0.07 ± 0.01**	56.3	0.01 ± 0.004	66.7	0.08 ± 0.02	50.0	0.05 ± 0.01	-66.7
1.0	0.45 ± 0.04	0.03 ± 0.02	0.17 ± 0.01**	62.2	0.002 ± 0.002**	93.3	0.1 ± 0.03**	77.8	0.07 ± 0.01	-133.3
10	1.8 ± 0.2	0.06 ± 0.01	0.80 ± 0.1**	55.6	0.0 ± 0.0*	100.0	0.36 ± 0.07**	80.0	0.15 ± 0.03	-150.0

* P <0.05; ** P <0.01

[a] Mean ± S.E.M.; [b] - indicates stimulation of product formation

Discussion

In these studies, we have investigated in detail the metabolism of CPA using the purified reconstituted P450 system. We have examined the five human P450s which have been previously reported to activate CPA via 4-hydroxylation as well as inactivate CPA via *N*-dechloroethylation (*16*). We also assessed these pathways with eight polymorphic variants (six variants of CYP2B6 and two of CYP2C9) to more fully characterize and elucidate potential differences in metabolism. To our knowledge, this is the most comprehensive evaluation of both the activation and inactivation pathways for CPA metabolism by human P450s. We have also measured the formation of the two predominant CPA metabolites by HLM and following inhibition of the specific P450s. These studies are the first to evaluate both the activation and inactivation pathways using drugs that are known P450 inhibitors.

These results demonstrate that CYP2B6 and CYP2C19 are the most efficient P450s tested for the activation of CPA. The K_m value observed for CYP2B6 (0.6 ± 0.1 mM) is slightly less than literature reports from supersomes (1.4 – 1.9 mM) and purified protein (3.6 mM) which may be due to our reaction conditions which were not identical to those used in previous studies (*3, 8, 32, 35, 44*). Our studies demonstrate that CYP2C19 is ~10-fold less efficient than CYP2B6, yet it is more efficient at activation than the other P450s tested. CYP2C19 expression in human liver is generally low, which suggests that its role in the activation of CPA may be minor (*45*). This is supported by a recent study using pooled HLM which revealed that inhibition of CYP2C19 (by (+)-*N*-3-benzylnirvanol) did not lead to a significant decrease in the activation of CPA (*35*). Yet, carriers of the non-functional *CYP2C19.2* allele had significantly reduced elimination of CPA, implicating CYP2C19 as being involved in CPA metabolism in some way (*46, 47*). Given the relatively high catalytic

efficiency of CYP2C19, it may be crucial under circumstances of inhibition of the other P450s involved in CPA metabolism or expression changes in the P450 profile, both of which commonly may occur in humans. The other P450s (CYP2C9, CYP3A4, and CYP3A5) were significantly less efficient at hydroxylating CPA in our reconstituted system (each of their efficiencies were ≤1). Our studies as well as others have demonstrated that CYP3A4 is responsible for at least 50% of CPA activation (*3, 8, 35*). This is most likely a result of the much higher expression level of CYP3A4 than CYP2B6 (*45*). The greatly increased amount of CYP3A4 compensates for the relatively low catalytic ability associated with the enzyme for this reaction. In addition, it is possible that other components in the previously used systems (HLM and rats), which are not present in our reconstituted system, may aid CYP3A4 in this reaction. The relatively low efficiency of CYP2C9 for CPA activation that we obtained supports the developing view that CYP2C9 contributions to CPA activation are minor and may be dependent on the relative concentrations of b5 and CPR (*2, 8, 35, 48*).

In coordinated studies, the P450s were evaluated for the inactivation of CPA. The inactive and toxic metabolites of CPA are substantially and variably produced in cancer patients (*49*). Only CYP2B6 and CYP3A4 have been previously tested for this reaction, thus we examined additional P450s that may also contribute to the elevation of CAA in humans. Our results confirm that only CYP3A4 catalyzes the CPA inactivation (*43, 50*). Furthermore, we observed that CYP3A4 is more efficient for this pathway (V_{max}/K_m = 7.5 $min^{-1}mM^{-1}$) than the activation pathway (V_{max}/K_m = 1 $min^{-1}mM^{-1}$). The reason for this is unknown and modeling the binding of CPA into CYP3A4 and other P450s did not provide significant structural insights that would have explained the preferred metabolic pathway of CYP3A4 (models not shown). None of the other P450s tested catalyzed the inactivation of CPA.

The polymorphic variants of CYP2B6 and CYP2C9 are known to exhibit altered catalytic activities for various substrates (*24, 27-29*). All of the tested CYP2B6 polymorphisms exhibited attenuated activation for CPA compared with wild type. The most common variants in the population, CYP2B6*4 and CYP2B6*6 (both have ~30% occurrence in some populations) exhibited very low levels of metabolism, only 2-3% of the total activation that is observed with wild type (*51-55*). The less common variants, CYP2B6*7 and CYP2B6*9 produced half of the acrolein that CYP2B6*1 produced (*55-57*). This suggests that carriers of these allelic variants may exhibit decreased CPA activation. Furthermore, it has been demonstrated that many of the polymorphic variants have lower expression levels than wild-type CYP2B6, which may significantly decrease CPA activation *in vivo* even more (*51, 58, 59*). Clinical studies with regards to CYP2B6 genotype and the therapeutic outcome of CPA are confounding (*60*). Nakajima, *et al.* found no association between CPA activation and *CYP2B6.6* and *CYP2B6.9* expression in patients. (*61*). Yet, in another study, heterozygous carriers of both of these alleles had elevated circulating levels of activated CPA (*30*). The discrepancy between these studies may be due to several factors including: participant number, CPA dose, or concurrent treatments of the patients with other drugs. In other work, *CYP2B6.2* and *CYP2B6.5* carriers had higher incidences of CPA associated toxicities and the *CYP2B6.2*, *CYP2B6.4*, *CYP2B6.8*, and *CYP2B6.9* alleles were associated with lowered overall survival of patients post-CPA treatment (*34*). Yet, multiple studies have failed to find a correlation between CYP2B6 genotype and therapeutic outcome following treatment with CPA (*46, 62*). The lack of CYP2B6 genotype effect on CPA pharmacokinetics is most likely due to the contributions of other P450s, such as CYP3A4, with regards to activation of CPA.

This study provides the first information on the inactivation of CPA by the polymorphic variants of CYP2B6 and CYP2C9. CYP2B6*4, CYP2B6*7, and CYP2B6*9 produced CAA (2 to 3-fold more than CYP2B6*1), albeit only 10% of the total CAA produced by CYP3A4 at the same CPA concentration. This indicates that the wild type, as well as the polymorphic variants of CYP2B6 and CYP2C9, do not greatly contribute to the production of the toxic metabolite CAA from CPA.

Given that CYP3A4-catalyzed metabolism of CPA results in significant CPA inactivation and production of toxic metabolites, we investigated CYP3A4 inhibition in pooled HLM to determine whether we could reduce the inactivation and enhance the activation of CPA. Ketoconazole-mediated inhibition of CYP3A4 in HLMs resulted in a near-complete loss of CPA inactivation and an ~50% reduction in CPA activation. Thus, CYP3A4 inhibition, although not totally beneficial in these studies, should not be ruled out as a potential method to maximize CPA's effectiveness and reduce its associated toxicities. Additional studies testing other CYP3A4 inhibitors or various concentrations of ketoconazole are planned, and may reveal a critical point in the balance of the two pathways that can be exploited for therapeutic purposes. With inhibition of CYP2B6, we observed a 50-80% reduction of CPA activation, similar to previous results in the literature (*4, 9, 35*). Interestingly, the inhibition of CYP2B6 by clopidogrel at a higher dose of CPA (10 mM) resulted in a >2-fold increase in CPA inactivation. This is most likely due to increased metabolism of CPA by CYP3A4 in the absence of CYP2B6 catalytic activity, thus underscoring the importance of CYP2B6-mediated metabolism of CPA for not only the metabolic activation but also for protecting against toxicity.

In summary, we have demonstrated that CYP2B6 and CYP2C19 are the most efficient enzymes for activation of CPA *in vitro*. We also determined that CYP3A4, while not kinetically efficient at this reaction, contributes to ~50% of the metabolism in HLM presumably due to the fact that CYP3A4 is expressed in significantly greater quantities than the other P450s in the liver. Importantly, we showed that all of the CYP2B6 polymorphisms exhibited reduced ability to activate CPA as compared to wild type and that none of the polymorphisms exhibited significantly altered amounts of CPA inactivation. This suggests that determining P450 genotypes in patients may prove to be very helpful to gain a full comprehension of the patients' response to CPA. Finally, we showed that manipulation of the two primary routes for CPA metabolism is possible through inhibition of the P450s in HLM. This warrants further exploration as it may be advantageous to favor CYP2B6-mediated CPA metabolism in patients by inhibiting CYP3A4 or inducing CYP2B6 activity.

References

1. Sladek, N. E. (1988) Metabolism of oxazaphosphorines, *Pharmacol Ther 37*, 301-355.

2. Chang, T. K., Weber, G. F., Crespi, C. L., and Waxman, D. J. (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes, *Cancer Res 53*, 5629-5637.

3. Huang, Z., Roy, P., and Waxman, D. J. (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide, *Biochem Pharmacol 59*, 961-972.

4. Ren, S., Yang, J. S., Kalhorn, T. F., and Slattery, J. T. (1997) Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethylcyclophosphamide in human liver microsomes, *Cancer Res 57*, 4229-4235.

5. Chen, C. S., Lin, J. T., Goss, K. A., He, Y. A., Halpert, J. R., and Waxman, D. J. (2004) Activation of the anticancer prodrugs cyclophosphamide and ifosfamide: identification of cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics, *Mol Pharmacol 65*, 1278-1285.

6. Brock, N., Hilgard, P., Peukert, M., Pohl, J., and Sindermann, H. (1988) Basis and new developments in the field of oxazaphosphorines, *Cancer Invest 6*, 513-532.

7. Roy, P., and Waxman, D. J. (2006) Activation of oxazaphosphorines by cytochrome P450: application to gene-directed enzyme prodrug therapy for cancer, *Toxicol In Vitro 20*, 176-186.

8. Roy, P., Yu, L. J., Crespi, C. L., and Waxman, D. J. (1999) Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles, *Drug Metab Dispos 27*, 655-666.

9. Yu, L. J., Drewes, P., Gustafsson, K., Brain, E. G., Hecht, J. E., and Waxman, D. J. (1999) In vivo modulation of alternative pathways of P-450-catalyzed cyclophosphamide metabolism: Impact on pharmacokinetics and antitumor activity, *J Pharmacol Exp Ther 288*, 928-937.

10. Aleksa, K., Ito, S., and Koren, G. (2004) Renal-tubule metabolism of ifosfamide to the nephrotoxic chloroacetaldehyde: pharmacokinetic modeling for estimation of intracellular levels, *J Lab Clin Med 143*, 159-162.

11. Dubourg, L., Michoudet, C., Cochat, P., and Baverel, G. (2001) Human kidney tubules detoxify chloroacetaldehyde, a presumed nephrotoxic metabolite of ifosfamide, *J Am Soc Nephrol 12*, 1615-1623.

12. Woodland, C., Ito, S., Granvil, C. P., Wainer, I. W., Klein, J., and Koren, G. (2000) Evidence of renal metabolism of ifosfamide to nephrotoxic metabolites, *Life Sci 68*, 109-117.

13. Hadidi, A. H., Coulter, C. E., and Idle, J. R. (1988) Phenotypically deficient urinary elimination of carboxyphosphamide after cyclophosphamide administration to cancer patients, *Cancer Res 48*, 5167-5171.

14. Nieto, Y., Xu, X., Cagnoni, P. J., Matthes, S., Shpall, E. J., Bearman, S. I., Murphy, J., and Jones, R. B. (1999) Nonpredictable pharmacokinetic behavior of high-dose cyclophosphamide in combination with cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea, *Clin Cancer Res 5*, 747-751.

15. Sladek, N. E. (1972) Therapeutic efficacy of cyclophosphamide as a function of inhibition of its metabolism, *Cancer Res 32*, 1848-1854.

16. Sladek, N. E. (1972) Therapeutic efficacy of cyclophosphamide as a function of its metabolism, *Cancer Res 32*, 535-542.

17. Tasso, M. J., Boddy, A. V., Price, L., Wyllie, R. A., Pearson, A. D., and Idle, J. R. (1992) Pharmacokinetics and metabolism of cyclophosphamide in paediatric patients, *Cancer Chemother Pharmacol 30*, 207-211.

18. Johnson, L. A., Malayappan, B., Tretyakova, N., Campbell, C., MacMillan, M. L., Wagner, J. E., and Jacobson, P. A. (2012) Formation of cyclophosphamide specific DNA adducts in hematological diseases, *Pediatr Blood Cancer 58*, 708-714.

19. Ayash, L. J., Wright, J. E., Tretyakov, O., Gonin, R., Elias, A., Wheeler, C., Eder, J. P., Rosowsky, A., Antman, K., and Frei, E., 3rd. (1992) Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response, *J Clin Oncol 10*, 995-1000.

20. Murdych, T., and Weisdorf, D. J. (2001) Serious cardiac complications during bone marrow transplantation at the University of Minnesota, 1977-1997, *Bone Marrow Transplant 28*, 283-287.

21. Bearman, S. I., Petersen, F. B., Schor, R. A., Denney, J. D., Fisher, L. D., Appelbaum, F. R., and Buckner, C. D. (1990) Radionuclide ejection fractions in the evaluation of patients being considered for bone marrow transplantation: risk for cardiac toxicity, *Bone Marrow Transplant 5*, 173-177.

22. Anderson, L. W., Chen, T. L., Colvin, O. M., Grochow, L. B., Collins, J. M., Kennedy, M. J., and Strong, J. M. (1996) Cyclophosphamide and 4-Hydroxycyclophosphamide/aldophosphamide kinetics in patients receiving high-dose cyclophosphamide chemotherapy, *Clin Cancer Res 2*, 1481-1487.

23. Chen, T. L., Passos-Coelho, J. L., Noe, D. A., Kennedy, M. J., Black, K. C., Colvin, O. M., and Grochow, L. B. (1995) Nonlinear pharmacokinetics of cyclophosphamide

in patients with metastatic breast cancer receiving high-dose chemotherapy followed by autologous bone marrow transplantation, *Cancer Res 55*, 810-816.

24. Zhang, H., Sridar, C., Kenaan, C., Amunugama, H., Ballou, D. P., and Hollenberg, P. F. (2011) Polymorphic variants of cytochrome P450 2B6 (CYP2B6.4 - CYP2B6.9) exhibit altered rates of metabolism for bupropion and efavirenz: A charge-reversal mutation in the K139E variant (CYP2B6.8) impairs formation of a functional P450-reductase complex, *J Pharmacol Exp Ther*.

25. Watanabe, T., Sakuyama, K., Sasaki, T., Ishii, Y., Ishikawa, M., Hirasawa, N., and Hiratsuka, M. (2010) Functional characterization of 26 CYP2B6 allelic variants (CYP2B6.2-CYP2B6.28, except CYP2B6.22), *Pharmacogenet Genomics 20*, 459-462.

26. Wei, L., Locuson, C. W., and Tracy, T. S. (2007) Polymorphic variants of CYP2C9: mechanisms involved in reduced catalytic activity, *Mol Pharmacol 72*, 1280-1288.

27. Rettie, A. E., Wienkers, L. C., Gonzalez, F. J., Trager, W. F., and Korzekwa, K. R. (1994) Impaired (S)-warfarin metabolism catalysed by the R144C allelic variant of CYP2C9, *Pharmacogenetics 4*, 39-42.

28. Crespi, C. L., and Miller, V. P. (1997) The R144C change in the CYP2C9*2 allele alters interaction of the cytochrome P450 with NADPH:cytochrome P450 oxidoreductase, *Pharmacogenetics 7*, 203-210.

29. Bumpus, N. N., and Hollenberg, P. F. (2008) Investigation of the mechanisms underlying the differential effects of the K262R mutation of P450 2B6 on catalytic activity, *Mol Pharmacol 74*, 990-999.

30. Xie, H., Griskevicius, L., Stahle, L., Hassan, Z., Yasar, U., Rane, A., Broberg, U., Kimby, E., and Hassan, M. (2006) Pharmacogenetics of cyclophosphamide in patients with hematological malignancies, *Eur J Pharm Sci 27*, 54-61.

31. Rocha, V., Porcher, R., Fernandes, J. F., Filion, A., Bittencourt, H., Silva, W., Jr., Vilela, G., Zanette, D. L., Ferry, C., Larghero, J., Devergie, A., Ribaud, P., Skvortsova, Y., Tamouza, R., Gluckman, E., Socie, G., and Zago, M. A. (2009) Association of drug metabolism gene polymorphisms with toxicities, graft-versus-host disease and survival after HLA-identical sibling hematopoietic stem cell transplantation for patients with leukemia, *Leukemia 23*, 545-556.

32. Xie, H. J., Yasar, U., Lundgren, S., Griskevicius, L., Terelius, Y., Hassan, M., and Rane, A. (2003) Role of polymorphic human CYP2B6 in cyclophosphamide bioactivation, *Pharmacogenomics J 3*, 53-61.

33. Takada, K., Arefayene, M., Desta, Z., Yarboro, C. H., Boumpas, D. T., Balow, J. E., Flockhart, D. A., and Illei, G. G. (2004) Cytochrome P450 pharmacogenetics as a predictor of toxicity and clinical response to pulse cyclophosphamide in lupus nephritis, *Arthritis Rheum 50*, 2202-2210.

34. Bray, J., Sludden, J., Griffin, M. J., Cole, M., Verrill, M., Jamieson, D., and Boddy, A. V. (2010) Influence of pharmacogenetics on response and toxicity in breast cancer patients treated with doxorubicin and cyclophosphamide, *Br J Cancer 102*, 1003-1009.

35. Raccor, B. S., Claessens, A. J., Dinh, J. C., Park, J. R., Hawkins, D. S., Thomas, S. S., Makar, K. W., McCune, J. S., and Totah, R. A. (2011) Potential contribution of cytochrome P450 2B6 to hepatic 4-hydroxycyclophosphamide formation: in vitro and in vivo investigations, *Drug Metab Dispos 40*, 54-63.

36. Jenkins, C. M., Pikuleva, I., and Waterman, M. R. (1998) Expression of eukaryotic cytochromes P450 in E. coli, *Methods in molecular biology 107*, 181-193.

37. Scott, E. E., Spatzenegger, M., and Halpert, J. R. (2001) A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function, *Arch Biochem Biophys 395*, 57-68.

38. Hanna, I. H., Roberts, E. S., and Hollenberg, P. F. (1998) Molecular basis for the differences in lidocaine binding and regioselectivity of oxidation by cytochromes P450 2B1 and 2B2, *Biochemistry 37*, 311-318.

39. Domanski, T. L., Finta, C., Halpert, J. R., and Zaphiropoulos, P. G. (2001) cDNA cloning and initial characterization of CYP3A43, a novel human cytochrome P450, *Mol Pharmacol 59*, 386-392.

40. Hustert, E., Haberl, M., Burk, O., Wolbold, R., He, Y. Q., Klein, K., Nuessler, A. C., Neuhaus, P., Klattig, J., Eiselt, R., Koch, I., Zibat, A., Brockmoller, J., Halpert, J. R., Zanger, U. M., and Wojnowski, L. (2001) The genetic determinants of the CYP3A5 polymorphism, *Pharmacogenetics 11*, 773-779.

41. Hummel, M. A., Locuson, C. W., Gannett, P. M., Rock, D. A., Mosher, C. M., Rettie, A. E., and Tracy, T. S. (2005) CYP2C9 genotype-dependent effects on in vitro drug-drug interactions: switching of benzbromarone effect from inhibition to activation in the CYP2C9.3 variant, *Mol Pharmacol 68*, 644-651.

42. Huang, Z., and Waxman, D. J. (1999) High-performance liquid chromatographic-fluorescent method to determine chloroacetaldehyde, a neurotoxic metabolite of the anticancer drug ifosfamide, in plasma and in liver microsomal incubations, *Anal Biochem 273*, 117-125.

43. Yu, L., and Waxman, D. J. (1996) Role of cytochrome P450 in oxazaphosphorine metabolism. Deactivation via N-dechloroethylation and activation via 4-hydroxylation catalyzed by distinct subsets of rat liver cytochromes P450, *Drug Metab Dispos 24*, 1254-1262.

44. Kumar, V., Rock, D. A., Warren, C. J., Tracy, T. S., and Wahlstrom, J. L. (2006) Enzyme source effects on CYP2C9 kinetics and inhibition, *Drug Metab Dispos 34*, 1903-1908.

45. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians, *J Pharmacol Exp Ther 270*, 414-423.

46. Timm, R., Kaiser, R., Lotsch, J., Heider, U., Sezer, O., Weisz, K., Montemurro, M., Roots, I., and Cascorbi, I. (2005) Association of cyclophosphamide pharmacokinetics to polymorphic cytochrome P450 2C19, *Pharmacogenomics J 5*, 365-373.

47. Helsby, N. A., Hui, C. Y., Goldthorpe, M. A., Coller, J. K., Soh, M. C., Gow, P. J., De Zoysa, J. Z., and Tingle, M. D. (2010) The combined impact of CYP2C19 and CYP2B6 pharmacogenetics on cyclophosphamide bioactivation, *Br J Clin Pharmacol 70*, 844-853.

48. Chang, T. K., Yu, L., Goldstein, J. A., and Waxman, D. J. (1997) Identification of the polymorphically expressed CYP2C19 and the wild-type CYP2C9-ILE359 allele as low-Km catalysts of cyclophosphamide and ifosfamide activation, *Pharmacogenetics 7*, 211-221.

49. Boddy, A. V., Furtun, Y., Sardas, S., Sardas, O., and Idle, J. R. (1992) Individual variation in the activation and inactivation of metabolic pathways of cyclophosphamide, *J Natl Cancer Inst 84*, 1744-1748.

50. Bohnenstengel, F., Hofmann, U., Eichelbaum, M., and Kroemer, H. K. (1996) Characterization of the cytochrome P450 involved in side-chain oxidation of cyclophosphamide in humans, *Eur J Clin Pharmacol 51*, 297-301.

51. Lang, T., Klein, K., Fischer, J., Nussler, A. K., Neuhaus, P., Hofmann, U., Eichelbaum, M., Schwab, M., and Zanger, U. M. (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver, *Pharmacogenetics 11*, 399-415.

52. Mehlotra, R. K., Ziats, M. N., Bockarie, M. J., and Zimmerman, P. A. (2006) Prevalence of CYP2B6 alleles in malaria-endemic populations of West Africa and Papua New Guinea, *Eur J Clin Pharmacol 62*, 267-275.

53. Davaalkham, J., Hayashida, T., Tsuchiya, K., Gatanaga, H., Nyamkhuu, D., and Oka, S. (2009) Allele and genotype frequencies of cytochrome P450 2B6 gene in a Mongolian population, *Drug Metab Dispos 37*, 1991-1993.

54. Jacob, R. M., Johnstone, E. C., Neville, M. J., and Walton, R. T. (2004) Identification of CYP2B6 sequence variants by use of multiplex PCR with allele-specific genotyping, *Clin Chem 50*, 1372-1377.

55. Guan, S., Huang, M., Li, X., Chen, X., Chan, E., and Zhou, S. F. (2006) Intra- and inter-ethnic differences in the allele frequencies of cytochrome P450 2B6 gene in Chinese, *Pharm Res 23*, 1983-1990.

56. Jinno, H., Tanaka-Kagawa, T., Ohno, A., Makino, Y., Matsushima, E., Hanioka, N., and Ando, M. (2003) Functional characterization of cytochrome P450 2B6 allelic variants, *Drug Metab Dispos 31*, 398-403.

57. Joy, M. S., La, M., Wang, J., Bridges, A. S., Hu, Y., Hogan, S. L., Frye, R. F., Blaisdell, J., Goldstein, J. A., Dooley, M. A., Brouwer, K. L., and Falk, R. J. (2012) Cyclophosphamide and 4-hydroxycyclophosphamide pharmacokinetics in patients with glomerulonephritis secondary to lupus and small vessel vasculitis, *Br J Clin Pharmacol 74*, 445-455.

58. Desta, Z., Saussele, T., Ward, B., Blievernicht, J., Li, L., Klein, K., Flockhart, D. A., and Zanger, U. M. (2007) Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro, *Pharmacogenomics 8*, 547-558.

59. Zanger, U. M., and Klein, K. (2013) Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance, *Frontiers in genetics 4*, 24.

60. Helsby, N. A., and Tingle, M. D. (2012) Which CYP2B6 Variants Have Functional Consequences for Cyclophosphamide Bioactivation?, *Drug Metab Dispos 40*, 635-637.

61. Nakajima, M., Komagata, S., Fujiki, Y., Kanada, Y., Ebi, H., Itoh, K., Mukai, H., Yokoi, T., and Minami, H. (2007) Genetic polymorphisms of CYP2B6 affect the pharmacokinetics/pharmacodynamics of cyclophosphamide in Japanese cancer patients, *Pharmacogenet Genomics 17*, 431-445.

62. Ekhart, C., Doodeman, V. D., Rodenhuis, S., Smits, P. H., Beijnen, J. H., and Huitema, A. D. (2008) Influence of polymorphisms of drug metabolizing enzymes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) on the pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide, *Pharmacogenet Genomics 18*, 515-523.

CHAPTER 3

HYDROXYLATION AND *N*-DECHLOROETHYLATION OF IFOSFAMIDE AND DEUTERATED IFOSFAMIDE BY THE CYTOCHROME P450S AND THEIR COMMON POLYMORPHIC VARIANTS

Introduction

Ifosfamide (IFO) is a DNA alkylating agent used in cancer chemotherapeutic regimens. It is the structural isomer of the more commonly used anti-neoplastic and immunosuppressive agent, cyclophosphamide (CPA) (*1*). Toxicities associated with IFO treatment limit its use, even though the drug has proven more effective than CPA in a wide range of malignant diseases (*2*-*7*). IFO-induced toxicities include moderate-to-severe nephrotoxicity, which occurs in ~30% of the patient population, and neurotoxicities, which occur in ~20% of the patient population (*8*-*10*). IFO treatment is also associated with life-threatening arrhythmias, heart failure and severe encephalopathy (*1*). IFO-associated toxicities are related to the metabolism of the drug; therefore, improving our knowledge of IFO metabolism may aid patient treatment and expand the use of IFO to a broader range of disease states and patient populations.

IFO is a prodrug which is activated via hydroxylation by cytochrome P450s (P450s) on the 4′ carbon (*11*). CYP2B6, CYP3A4, and CYP3A5 are the primary P450s responsible for the activation of IFO. However, CYP2C9 and CYP2C19 may also play a role (*12, 13*). After hydroxylation, IFO undergoes a series of spontaneous events to produce the active metabolite, ifosfamide mustard and equimolar amounts of acrolein (Figure 1.7). In addition, IFO may be *N*-dechloroethylated by CYP2B6, CYP3A4, and CYP3A5 (*12, 13*), leading to the inactivation of IFO and formation of the nephro- and neurotoxic metabolites, 2-dechloroethylifosfamide or 3-dechloroethylifosfamide and equimolar amounts of chloroacetaldehyde (CAA) (See Figure 1.7 to clarify 2-dechloroethylifosfamide versus 3-dechloroethylifosfamide). Figure 1.7 depicts the proposed mechanism for IFO metabolism. It is estimated that 25-60% of IFO is *N*-dechloroethylated in humans whereas only 10% of CPA undergoes this reaction (*14-16*). It has been suggested that elevated circulating levels of CAA in IFO-treated patients are responsible for the development of IFO-specific nephrotoxicity (*17-19*). Yet, *in vitro* studies have demonstrated that the minimum toxic concentration of CAA is 500 μM, which is well above the circulating levels of CAA in IFO-treated patients (0.5 – 109 μM) (*18, 20-22*). Thus, organ-specific toxicities are likely due to *N*-dechloroethylation of IFO at the organ of interest, which would increase local tissue concentrations of CAA to potentially toxic levels (*10, 17*). Tissue expression of the P450s is highly variable; for instance, in the kidney CYP3A5 is highly expressed, while CYP3A4 is not (*23-25*). Therefore, CYP3A5 has been suggested to play a more prominent role than other P450s in IFO-induced nephrotoxicity. Our studies have focused on characterizing the catalytic abilities of the P450s to ascertain the likelihood of specific P450 involvement in IFO metabolism.

Several studies suggest that CAA is primarily responsible for IFO-induced toxicities (*18, 19*). Therefore, suppression of CAA formation by favoring hydroxylation over *N*-dechloroethylation of IFO may improve efficacy and reduce adverse events. Previously this approach has been referred to as "metabolic switching" (*26*). In this report, we have examined the ability to initiate metabolic switching of IFO. The metabolism of deuterated IFO (d4IFO) was compared with the metabolism of IFO *in vitro* (Figure 3.1). We demonstrated that deuteration of IFO resulted in enhanced hydroxylation and reduced *N*-dechloroethylation for all of the P450s tested (CYP2B6, CYP2C9, CYP2C19, and CYP3A4). Although the differences between d4IFO metabolism and IFO metabolism were moderate, a trend towards metabolic switching is apparent. In order to thoroughly characterize IFO and d4IFO metabolism, the polymorphic variants of CYP2B6 (CYP2B6*4, CYP2B6*6-*9) and CYP2C9 (CYP2C9*2 and CYP2C9*3) were assessed for hydroxylation and *N*-dechloroethylation of IFO and d4IFO. These studies revealed differences in the catalytic rates of the polymorphic variants for both pathways and for both IFO and d4IFO. In brief, CYP2B6*4 and CYP2B6*6 exhibit decreased activation and inactivation for the drugs while CYP2B6*7 and CYP2B6*9 exhibit increased activation and inactivation as compared to the wild type enzyme. d4IFO was more readily activated by the variants than IFO without being inactivated (d4IFO inactivation was only observed with wild type CYP2B6). The differences observed for the CYP2C9 variants were very small. These findings may relate to patient outcomes with IFO treatment, since CYP2B6 is largely responsible for the inactivation of IFO *in vivo* (*27*).

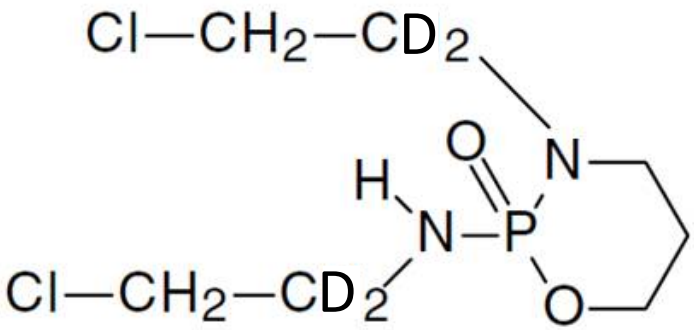


Figure 3.1 - Chemical structure of deuterated-IFO. IFO was synthesized with deuterium in the indicated positions. *N*-dechloroethylation of d4IFO by the P450s should be inhibited as compared to *N*-dechloroethylation of IFO and therefore, the hydroxylation of d4IFO at the 4′ position by the P450s is enhanced.

Experimental Procedures

Materials. NADPH, catalase, IFO, L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, L-α-phosphatidylserine, acrolein, CAA, 3-aminophenol, and adenosine were all purchased from Sigma Aldrich (St. Louis, MO). Pooled HLM were purchased from BD Biosciences (San Jose, CA). d4IFO was a gift from Dr. Susan Ludeman (Albany College of Pharmacy and Health Sciences) and Dr. M Eileen Dolan (University of Chicago). All other reagents were reagent grade and purchased from commercial sources.

cDNA expression and purification of Human P450s, P450 Polymorphic Variants, and Rat Cytochrome P450 Reductase. Polymorphic variants of CYP2B6 (CYP2B6*4, CYP2B6*6– CYP2B6*9) were over-expressed in *E.* coli C41 (DE3) cells as described previously (*28*). All P450s were expressed as their N-terminally truncated forms to increase the expression yield (*29, 30*). Rat CPR was expressed and purified as previously described (*31*). CYP3A5 was a gift from Dr. James Halpert (UCSD), and CYP2C9*2 and CYP2C9*3 were gifts from Dr. Tim Tracy (University of Kentucky) (*32-34*).

IFO and d4IFO Metabolism Assay. The metabolism of IFO and d4IFO by the human P450s was carried out in a reconstituted system. The reconstituted system consisting of P450 and CPR at a 1:2 molar ratio was incubated for 30 min at 20°C. Dilauroylphosphatidylcholine was not used in the reconstituted systems containing CYP2B6, CYP2C9, and CYP2C19 because it was not required for catalysis (data not shown). For the reactions catalyzed by CYP3A4 and CYP3A5, a mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine (1:1:1) were included in the reconstituted system for optimal activity. For these studies, 60 μg of the lipid mixture

was added to the proteins and the combination was incubated for 45 min at 4°C prior to use. The final reaction mixtures contained 1 μM P450, 2 μM CPR, 30 mM potassium phosphate (pH 7.4), 15 units of catalase, and 0-5.0 mM IFO or d4IFO in a total volume of 95 μL. The reaction mixtures were incubated at 37°C for 4 min followed by the addition of 5 μL of NADPH (final concentration of 1 mM) to initiate the reaction. After incubation at 37°C for 30 min, the reactions were terminated by the addition of 40 μL of ice-cold 5.5% zinc sulfate, 40 μL of ice-cold saturated barium hydroxide, and 20 μL of ice-cold 0.01M HCl as reported previously (*35*). The samples were centrifuged for 10 min at 13,200 rpm and kept on ice prior to derivatization and quantification by HPLC.

IFO Metabolism by Pooled Human Liver Microsomes. Metabolism of IFO by HLM was determined as described above with the substitution of 0.2 mg/mL of HLM for the reconstituted protein mixture. To specifically inhibit CYP3A4, 5 μM ketoconazole was introduced to the reaction mixtures prior to initiating the reaction with NADPH. Similarly, to inhibit CYP2B6, 5 μM clopidogrel was added to the reaction mixtures prior to initiating the reaction.

HPLC Analysis of IFO Metabolites. Acrolein was analyzed by HPLC; specifically 60 μL of the reaction mixture containing the acrolein product was incubated with 40 μL of fluorescence reagent (30 mg 3-aminophenol and 30 mg hydroxylamine HCl dissolved in 5 mL 1N HCl) for 25 min at 90°C to form the fluorescent product 7-hydroxyquinoline. The derivatized samples were then separated on an Agilent C_{18} column (Zorbax SB, 3.0 x 150 mm, 5 μm, Agilent Technologies), using a Shimadzu HPLC system. 7-Hydroxyquinoline

was eluted isocratically with 20% methanol in 0.1% phosphoric acid at a flow rate of 0.5 mL/min. Elution of 7-hydroxyquinoline occurred at 3.2 min as monitored fluorometrically at 515 nm with excitation of 350 nm (*35*). Calibration curves for acrolein quantification were generated by incubation of acrolein standards in the reaction mixtures containing no substrate or NADPH. The standard curve was linear in the range of 0-100 μM acrolein.

To detect CAA, 60 μL of the reaction mixture was incubated with 10 μL of 100 mM adenosine in 0.25 N HCl and 10 μL of 2 M sodium acetate (pH 4.5) for 2.5 hours at 80°C. Under these conditions, CAA was derivatized to the fluorescent product, 1-*N*-ethenoadenosine (*35*). The derivatized sample was then resolved on an Agilent C_{18} column (Zorbax SB, 3.0 x 150 mm, 5 μM, Agilent Technologies) with a mobile phase of 20% methanol in water, eluted isocratically with a flow rate of 0.5 mL/min. 1-*N*-ethenoadenosine was monitored fluorometrically at 411 nm with excitation at 270 nm. The derivatized product eluted at 12.7 min. Calibration curves for CAA quantification were generated by incubation of CAA standards in reaction mixtures containing no substrate or NADPH. The standard curve was linear from 0-150 μM CAA.

Data Analysis. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc.). The parameters for the enzyme kinetics were determined using non-linear regression analysis, specifically Michaelis-Menten analysis in GraphPad Prism. Statistical significance was determined using an unpaired t-test in GraphPad Prism.

Results

Metabolism of Ifosfamide and Deuterated Ifosfamide by Wild Type Human Cytochrome P450s. The capacity of the wild type P450s (CYP2B6, CYP2C9, CYP2C19, CYP4A4, and CYP3A5) to catalyze the activation of IFO and d4IFO was investigated in the purified reconstituted system. Figure 3.2 demonstrates the kinetic data generated using CYP3A4. Similar data were obtained for most of the other human P450s; these data are not shown, but they are summarized in Table 3.1. Of the P450s tested, only CYP2C9 exhibited no activity for IFO metabolism. Previous reports have indicated that CYP3A4 is the most efficient P450 for the activation of IFO (*12, 13*). However our, results suggest that CYP3A4 has moderate activity for this reaction (V_{max} /K_m = 0.4 $min^{-1}mM^{-1}$) (Figure 3.2 and Table 3.1). CYP3A5 exhibited the highest catalytic efficiency for the activation of IFO (V_{max} /K_m = 2.5 $min^{-1}mM^{-1}$) although it had the lowest V_{max} value (0.1 pmol/min/pmol P450). CYP2C19 was the next-most efficient P450 for the activation of IFO (V_{max} /K_m = 0.9 $min^{-1}mM^{-1}$). CYP2B6 exhibited the largest V_{max} (3.8 pmol/min/pmol P450), which is ~38-fold higher than that of CYP3A5. Similar kinetics were observed for d4IFO (Table 3.1 and Figure 3.2). Most notably, the K_m for activation by CYP2B6 was lower for d4IFO than IFO, resulting in a more efficient reaction. Similarly the K_m of CYP3A4 for d4IFO activation was reduced by a factor of 3, resulting in a more efficient reaction as well.

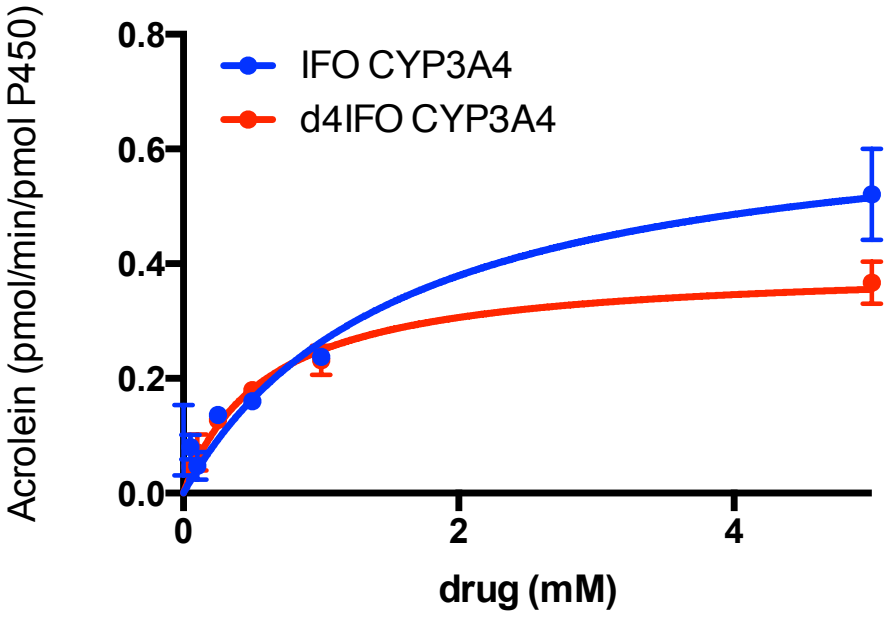


Figure 3.2 - Steady state kinetics for the activation of IFO and d4IFO by CYP3A4. IFO (blue) or d4IFO (red) (0.1 – 5.0 mM) was incubated in the reconstituted system containing 1 μM CYP3A4 for 30 min at 37°C. The details of the analytical methods are provided in the *Materials and Methods*. Shown are the nonlinear fits for the activation of the drugs determined using the Michaelis-Menten equation with Prism 6 (GraphPad Software, Inc.). The K_m and V_{max} values for all of the P450s tested are summarized in Table 3.1.

Table 3.1 - Kinetic Parameters for the Metabolism of IFO and d4IFO by Several Human P450s. The K_m and V_{max} values for CYP3A4 were obtained by fitting the data shown in Figure 3.2 to the Michaelis-Menten equation as described under *Materials and Methods*. Similar data and fits were produced and the same analysis was performed for each of the P450s indicated in this table. The reported values are averages that were determined from experiments performed in triplicate.

P450	Activation					
	IFO			d4IFO		
	V_{max} [a]	K_m [a]	V_{max}/K_m	V_{max} [a]	K_m [a]	V_{max}/K_m
	pmol/min/pmol P450	*mM*	$min^{-1}mM^{-1}$	*pmol/min/pmol P450*	*mM*	$min^{-1}mM^{-1}$
CYP2B6	3.8 ± 1	4.6 ± 2	0.8	4.1 ± 0.4	2.0 ± 0.4	2
CYP2C9	no acrolein formed		0	no acrolein formed		0
CYP2C19	1.0 ± 0.2	1.1 ± 0.6	0.9	1.0 ± 0.1	1.2 ± 0.4	0.8
CYP3A4	0.7 ± 0.1	1.6 ± 0.6	0.4	0.4 ± 0.03	0.6 ± 0.12	0.7
CYP3A5	0.1 ± 0.01	0.04 ± 0.03	2.5	not tested		N/A
P450	Inactivation					
	IFO			d4IFO		
	V_{max} [a]	K_m [a]	V_{max}/K_m	V_{max} [a]	K_m [a]	V_{max}/K_m
	pmol/min/pmol P450	*mM*	$min^{-1}mM^{-1}$	*pmol/min/pmol P450*	*mM*	$min^{-1}mM^{-1}$
CYP2B6	0.4 ± 0.04	2.0 ± 0.4	0.2	0.2 ± 0.05	2.0 ± 0.8	0.1
CYP2C9	no CAA formed		0	no CAA formed		0
CYP2C19	no CAA formed		0	no CAA formed		0
CYP3A4	0.14 ± 0.03	3.5 ± 1.7	0.04	0.05 ± 0.02	1.7 ± 1.2	0.03
CYP3A5	0.02 ± 0.005	0.4 ± 0.3	0.05	not tested		N/A

[a] Mean ± S.E.M.

N/A, CYP3A5 was not assessed for d4IFO metabolism

The amount of inactivation of IFO and d4IFO by P450s was investigated in the same samples described above and evaluated for the production of the inactive metabolite CAA (Table 3.1). CYP2C9 and CYP2C19 did not catalyze the inactivation of either IFO or d4IFO. CYP2B6 was approximately 4-fold more efficient at the inactivation of IFO than any of the other P450s tested, and this held true for d4IFO as well (Table 3.1). Interestingly, the V_{max} values for the inactivation of d4IFO by CYP2B6 and CYP3A4 were reduced compared with IFO, resulting in reduced efficiency for inactivation of d4IFO compared to IFO for these P450s.

Metabolism of Ifosfamide and Deuterated Ifosfamide by the Polymorphic Variants of CYP2B6 and CYP2C9. The polymorphic variants of CYP2B6 and CYP2C9 were examined for the activation of IFO and d4IFO. The polymorphic variants exhibited variable activation of IFO (Figure 3.3A). CYP2B6*7 and CYP2B6*9 produced nearly 3-fold more acrolein than CYP2B6*1 at 1 mM IFO. However, the common polymorphic variants CYP2B6*4 and CYP2B6*6 produced approximately half of the acrolein that wild type CYP2B6 produced. With regards to the activation of d4IFO, the polymorphic variants followed a trend similar to that observed for the activation of IFO but consistently produced more of the activated metabolite from d4IFO than from IFO (Table 3.2). For CYP2B6*1 and CYP2B6*7 this enhancement was statistically significant. The variants of CYP2C9 exhibited small variations in catalyzing IFO activation. As with the CYP2B6 variants, the activation of d4IFO by CYP2C9*2 and CYP2C9*3 followed the same pattern of variation as for IFO but with significantly higher levels of activated metabolite produced (Table 3.2).

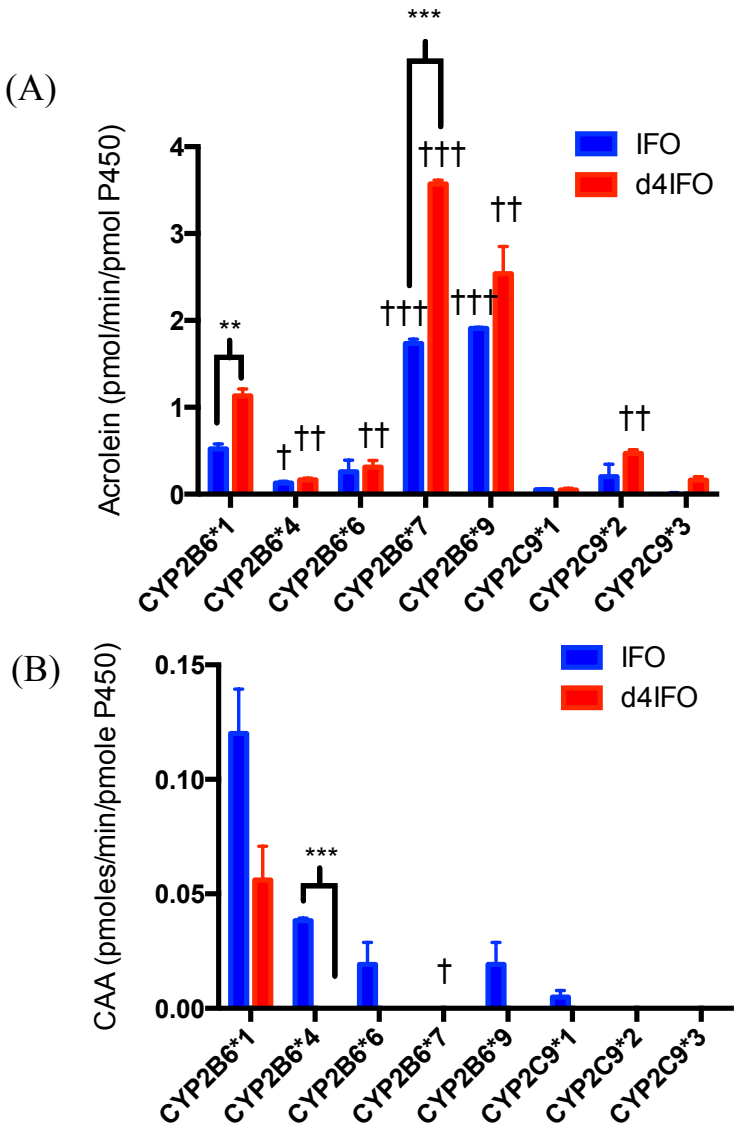


Figure 3.3 - Metabolism of IFO and d4IFO by the polymorphic variants of CYP2B6 and CYP2C9. IFO (blue) or d4IFO (red) (1 mM) was incubated in the reconstituted system with 1 µM P450 for 30 min at 37°C. Half of each sample was analyzed for acrolein to determine the rate of activation of IFO for each of the polymorphic variants (A). The other half of each sample was analyzed for CAA to determine the rate of inactivation of IFO for each of the polymorphic variants (B). The details of the analytical methods are provided in the *Materials and Methods*. The average for each experimental condition that was performed in triplicate is shown. Statistical significance was determined using an unpaired t-test in Prism 6 (GraphPad Software, Inc.). † P <0.05; ††P <0.01; ††† P ,0.005 for comparisons within polymorphic variants; * P <0.05; ** P <0.01; *** P <0.005 for comparisons between IFO and d4IFO. The data are summarized in Table 3.2.

Table 3.2 - The Metabolism of IFO and d4IFO by the Polymorphic Variants of CYP2B6 and CYP2C9. The data shown in Figure 3.4 are summarized here. Data are expressed as metabolite formation in pmol/min/pmol P450, and the metabolite production compared to wild type metabolite production in terms of fold change. Also, the percent increase or decrease of metabolite produced for d4IFO compared to IFO is given. The data were determined in triplicate. Statistical significance was determined using the unpaired t-test in Prism 6 (GraphPad Inc.).

P450	Activation				
	IFO		d4IFO		
	Acrolein Produced[a]	fold change over wild type	Acrolein Produced[a]	fold change over wild type	increase in active metabolite over IFO
	pmol/min/pmol P450		*pmol/min/pmol P450*		*%*
CYP2B6*1	0.5 ± 0.06	1.0	1.1 ± 0.08**	1.0	120.0
CYP2B6*4	0.1 ± 0.02†	0.2	0.2 ± 0.02††	0.2	100.0
CYP2B6*6	0.3 ± 0.1	0.6	0.3 ± 0.08††	0.3	0.0
CYP2B6*7	1.7 ± 0.05†††	3.4	3.6 ± 0.04†††***	3.3	111.8
CYP2B6*9	1.6 ± 0.3†††	3.2	2.5 ± 0.3††	2.3	56.3
CYP2C9*1	0.05 ± 0.01	1.0	0.05 ± 0.02	1.0	0.0
CYP2C9*2	0.2 ± 0.1	4.0	0.5 ± 0.05††	10.0	150.0
CYP2C9*3	0 ± 0	0.0	0.2 ± 0.04	4.0	N/A
P450	Inactivation				
	IFO		d4IFO		
	CAA Produced[a]	fold change over wild type	CAA Produced[a]	fold change over wild type	decrease in inactive metabolite over IFO
	pmol/min/pmol P450		*pmol/min/pmol P450*		*%*
CYP2B6*1	0.1 ± 0.02	1.0	0.06 ± 0.02	1.0	40.0
CYP2B6*4	0.04 ± 0.001	0.4	0 ± 0***	0.0	100.0
CYP2B6*6	0.02 ± 0.01	0.2	0 ± 0	0.0	100.0
CYP2B6*7	0 ± 0†	0.0	0 ± 0	0.0	N/A
CYP2B6*9	0.02 ± 0.01	0.2	0 ± 0	0.0	100.0
CYP2C9*1	0.01 ± 0.003	1.0	0 ± 0	1.0	100.0
CYP2C9*2	0 ± 0	0.0	0 ± 0	0.0	N/A
CYP2C9*3	0 ± 0	0.0	0 ± 0	0.0	N/A

† P <0.05; ††P <0.01; ††† P ,0.005 for comparisons within polymorphic variants
* P <0.05; ** P <0.01; *** P <0.005 for comparisons between IFO and d4IFO
[a] Mean ± S.E.M.
N/A no metabolite was detected with IFO, therefore percent change could not be calculated.

he inactivation of IFO and d4IFO by these polymorphic variants was also assessed using the same samples discussed above. All of the polymorphic variants of CYP2B6 tested produced less of the inactivated metabolite than wild type CYP2B6 (Figure 3.3B). In fact, the inactivation pathway of d4IFO by the CYP2B6 variants was essentially abolished. CYP2C9 catalyzed the inactivation IFO to a small extent, but its variants also failed to produce inactive metabolites for both IFO and d4IFO (Table 3.2).

Metabolism of Ifosfamide by Human Liver Microsomes. IFO activation by HLM was determined at concentrations of 1.0 and 10.0 mM IFO (Figure 3.4A). These concentrations of IFO were chosen to ensure metabolism by the respective P450s, based on the K_m values determined in Table 3.1. Acrolein production increased with increasing concentrations of IFO, as expected. The inhibition of CYP3A4 by ketoconazole reduced IFO activation by approximately 85% at both IFO concentrations. Inhibition of CYP2B6 by clopidogrel inhibited the activation of IFO by 67% and 47% at 1 mM and 10 mM IFO, respectively (Table 3.3).

IFO inactivation by HLM was also monitored. Incubation with ketoconazole resulted in a decrease of almost 90% in CAA production at both IFO concentrations (Figure 3.4B). Inhibition of CYP2B6 by clopidogrel decreased the inactivation of IFO by approximately 40% at both concentrations (Table 3.3).

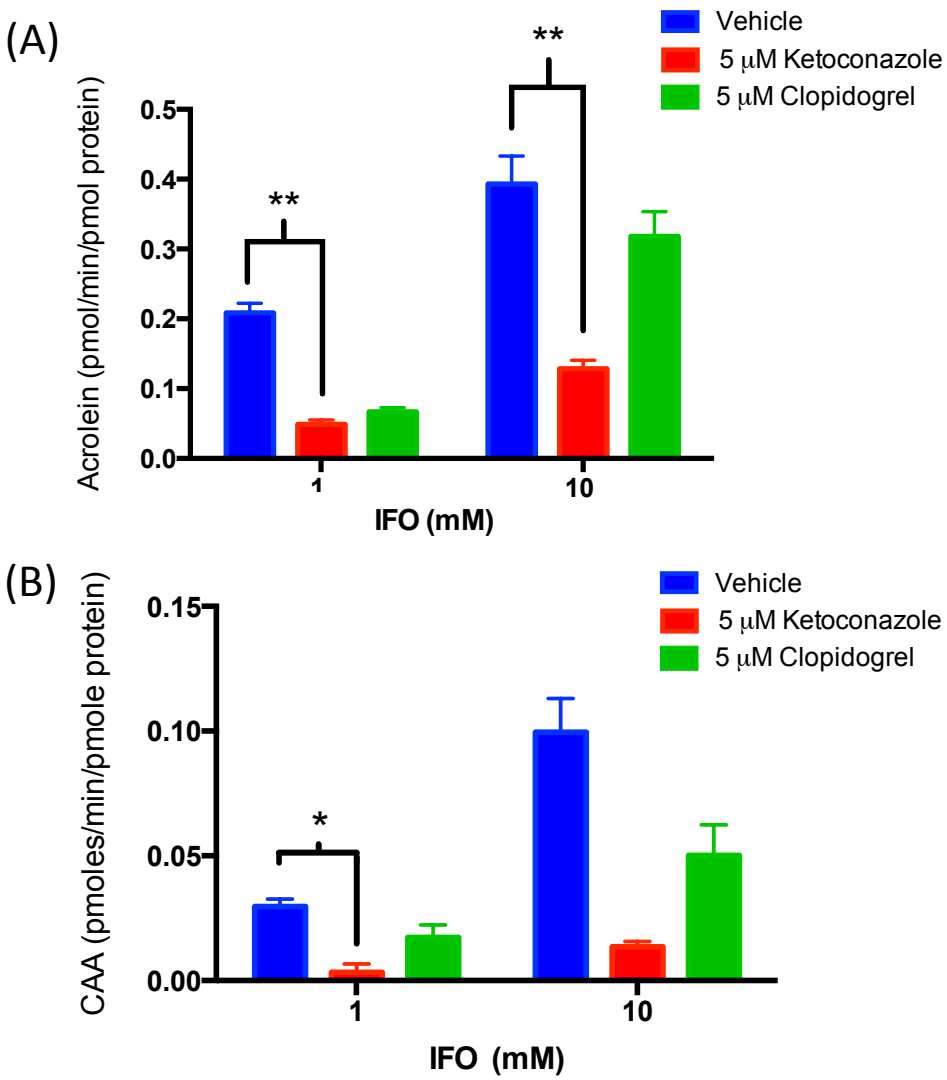


Figure 3.4 - Inhibition of IFO metabolism by HLMs in the presence of P450 inhibitors. IFO (1.0 or 10 mM) was incubated with HLM (0.2 mg/ mL). A short preincubation with 5 μM ketoconazole or 5 μM clopidogrel was used to inhibit CYP3A4 or CYP2B6, respectively. The total amount of acrolein production was measured to determine the effect of P450 inhibition on IFO activation (A). Similarly, the total amount of CAA production was measured to determine the effect of inhibition of CYP3A4 or CYP2B6 on the inactivation of IFO (B). The analytical methods are described in *Materials and Methods*. The experiments were performed twice in triplicate and the statistical significance was determined using an unpaired t-test in Prism 6 (GraphPad Software, Inc.). * $P < 0.05$; ** $P < 0.01$.

Table 3.3 - Summary of IFO metabolism by HLM in the presence of P450 inhibitors. The total amounts of acrolein or CAA produced by each of the experimental conditions are shown. Additionally, the percent inhibition for each of the metabolites in the ketoconazole-treated and clopidogrel-treated samples has been calculated. Note that the formation of CAA in the clopidogrel-treated samples increased. Statistical significance was determined using the unpaired t-test (P value < 0.05) in Prism 6 (GraphPad Inc.).

IFO (mM)	Activation				
	Vehicle Treated	5 uM Ketoconazole Treated		5 uM Clopidogrel Treated	
	Acrolein Formed[a]	Acrolein Formed[a]	Inhib	Acrolein Formed[a]	Inhib
	pmol/min^{-1}/pmol P450	*pmol/min^{-1}/pmol P450*	*%*	*pmol/min^{-1}/pmol P450*	*%*
1.0	0.1 ± 0.003	0.02 ± 0.001**	85.8	0.04 ± 0.01	67.4
10	0.4 ± 0.05	0.06 ± 0.005**	86.8	0.2 ± 0.01	47.1

IFO (mM)	Inactivation				
	Vehicle Treated	5 uM Ketoconazole Treated		5 uM Clopidogrel Treated	
	CAA Formed[a]	CAA Formed[a]	Inhib	CAA Formed[a]	Inhib
	pmol/min^{-1}/pmol P450	*pmol/min^{-1}/pmol P450*	*%*	*pmol/min^{-1}/pmol P450*	*%*
1.0	0.03 ± 0.003	0.003 ± 0.003*	88.7	0.02 ± 0.005	41.5
10	0.1 ± 0.01	0.014 ± 0.002	86.3	0.05 ± 0.01	49.6

* P <0.05; ** P <0.01

[a] Mean ± S.E.M.

Discussion

In order to enhance our understanding of the metabolism of IFO, we have used a purified recombinant system to assess the contributions of CYP2B6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 to the hydroxylation and the *N*-dechloroethylation of the drug. The polymorphic variants of CYP2B6 and CYP2C9 were also investigated in the reconstituted system to examine potential differences in the metabolism of IFO by the variants. We also investigated deuterium isotope effects on the hydroxylation and *N*-dechloroethylation of IFO. Michaelis-Menten kinetic values have been determined for all of the wild type P450s in the reconstituted system and therefore, accurate comparisons between the efficiencies of the enzymes for the activation and inactivation of both IFO and d4IFO is possible. This is the first time that CYP2C9, CYP2C19, and CYP3A5 have been evaluated for both reactions in this manner. Additionally, this is the first investigation of the metabolism of IFO by the polymorphic variants of CYP2B6 and CYP2C9.

The K_m values for the CYP2B6- and CYP3A4-mediated activation of IFO are similar to previous reports (Table1) (*12, 13*). In our hands, CYP2B6 yielded a larger V_{max} than that of CYP3A4, resulting in a higher efficiency for this reaction (V_{max}/K_m for CYP2B6 = 0.8 $min^{-1}mM^{-1}$ versus 0.4 $min^{-1}mM^{-1}$ for CYP3A4). This observation is in contrast with previous studies that analyzed the activation of IFO in Supersomes, where it was found that CYP3A4 was the most efficient enzyme for this reaction (*12, 13*). This discrepancy is most likely a result of the different expression systems used in the two studies and may possibly be due to the effect of b5 which was present in the previous studies, but not in our experiments. In our preliminary optimization experiments we examined the effect of b5 by adding it to the

reconstitution mixture at a range of ratios (0.5:1.0:0.25 to 0.5:1.0:2.0; P450:CPR:b5). We observed very little enhancement in the activation of IFO for all of the P450s, with the exception of CYP3A4, where no change was detected (data not shown). Therefore, b5 was not included in our optimized studies. Of the P450s tested, CYP3A5 had the lowest K_m (0.04 mM) and the highest efficiency for the activation of IFO (2.5 $min^{-1}mM^{-1}$). However, in patients, the plasma concentrations of IFO are well above 0.04 mM (up to ~1 mM) (*36, 37*). Given the low K_m of CYP3A5, the contributions of CYP3A5 to IFO metabolism may be important inside tissues or tumors where the concentration of IFO is thought to be considerably less than that in the plasma due to the relatively low cell membrane permeability of IFO. Additionally, CYP3A5 may be therapeutically important for IFO activation since it is ubiquitously expressed and therefore has the potential to activate IFO at the site of drug action versus in off-target tissues (*17, 23, 25*).

Intriguingly, the importance of CYP2C19 in the metabolism of IFO may have been overlooked previously. In our hands, CYP2C19 is the second-most efficient enzyme for IFO activation. Also CYP2C19 does not catalyze the inactivation of IFO, rendering it an ideal P450 for the metabolism of IFO. In Supersomes expressing CYP2C19, the activation of IFO (at 0.25 mM) was observed but, it was less than that by CYP2B6 or CYP3A4 in the same system (*12*). However, in the study using Supersomes did not determine the steady state kinetics for the enzymes that confirms the enzyme's capacity to efficiently catalyze the activation of IFO. Therefore, we believe CYP2C19 may be important for the metabolism of IFO given its high catalytic activity for activation of IFO.

With respect to the inactivation of IFO, only CYP2B6, CYP3A4, and CYP3A5 contribute to this pathway. The inactivation of IFO by CYP3A4 and CYP3A5 has been

extensively studied by McCune, et al. and our results support their observation that CYP3A4 and CYP3A5 have similar catalytic activities for IFO inactivation (*10*). However, their studies failed to address the inactivation of IFO by CYP2B6, which we found to be approximately 4-fold more efficient than CYP3A4 and CYP3A5. Introducing b5 into the recombinant mixture (at ratios in the range of 0.5:1:0.25 – 0.5:1:2; P450:CPR:b5) somewhat enhanced production of the inactive metabolite for all of the P450s, with the exception of CYP3A4, which showed no enhancement (data not shown). These findings agree with previous observations that CYP3A5-mediated IFO inactivation was enhanced by the addition of purified b5 to the reconstitution mixture, but that the CYP3A4-mediated inactivation of IFO was not (*10*).

Our studies on d4IFO metabolism demonstrated that deuteration of IFO enhanced the activation of IFO by CYP2B6 and CYP3A4. This is most likely due to the inhibition of *N*-dechloroethylation caused by the presence of deuterium on the chloroethyl groups of IFO (Figure 3.1). This hypothesis was supported by our findings in that the V_{max} values for the inactivation of d4IFO were lower than for the inactivation of IFO for all of the P450s tested. The efficiencies for the activation and inactivation of IFO and d4IFO by each of the tested P450s was compared. The overall improvement in the metabolism of IFO is best observed with CYP2B6, where the activation of d4IFO (V_{max}/K_m = 2 $min^{-1}mM^{-1}$) was ~2-fold more efficient than IFO (V_{max}/K_m = 0.8 $min^{-1}mM^{-1}$) and the inactivation of d4IFO (V_{max}/K_m = 0.1 $min^{-1}mM^{-1}$) was approximately 50% less efficient than for IFO (V_{max}/K_m = 0.2 $min^{-1}mM^{-1}$). Thus for the CYP2B6-mediated metabolism of d4IFO we observe an overall change of approximately 4-fold in the ratio of activation to inactivation which results in a significant enhancement of IFO metabolism. The deuterium isotope effects discussed here could prove

to be extremely beneficial for drug design and development studies. These studies demonstrate that utilizing deuterated drugs for "metabolic switching" may be a viable tool to enhance the therapeutic efficacy and reduce deleterious effects of IFO. Typically, 25-60% of IFO is metabolized to the inactivated and toxic metabolites dechloroethylifosfamide and chloroacetaldehyde. However, utilizing d4IFO may significantly reduce the formation of these toxic metabolites (*14-16*). Additional studies with d4IFO to address the effect of the deuterium isotopes on the DNA alkylating properties of the phosphoramide mustard of IFO merit further investigation.

The polymorphic variants of CYP2B6 and CYP2C9 exhibit altered catalytic activities for both the activation and inactivation of IFO and d4IFO as compared with the wild type. The common polymorphic variants CYP2B6*4 (K262R) and CYP2B6*6 (Q172H/K262R) showed reduced metabolism of IFO, with both the activation and inactivation pathways for IFO being less than those observed for CYP2B6*1. CYP2B6*4 and CYP2B6*6 are the two most commonly expressed polymorphisms of CYP2B6 in patient populations (>30% frequency for both variants in most populations) (*38-42*). The less common polymorphic variants CYP2B6*7 (Q172H/K262R/R487C) and CYP2B6*9 (Q172H) produced more of the activated metabolite of IFO and less of the inactivated metabolite of IFO than the wild type enzyme. This is interesting in light of studies performed with the structural analog CPA, where Ariyoshi et al. demonstrated that CYP2B6*4 had reduced activation and conversely CYP2B6*6 had enhanced activation of CPA (*43*). The authors concluded that the Q172R variant is capable of reinstating the activity that was lost by the introduction of the K262R mutation. With IFO this was not the case, but the addition of a third point mutant (R487C in CYP2B6*7) did fully reverse the effects of the first two mutations. This is the first evidence

that the insertion of a third point mutation in CYP2B6 can reverse the direction of the effect caused by the Q172H and K262R mutations. The polymorphic variant CYP2B6*8 was determined to be catalytically inactive with IFO, as expected (data not shown) (*28*). The polymorphic variants of CYP2C9 exhibited relatively small variations in catalytic efficiency compared with CYP2C9*1. In HLM, McCune et al. reported that expression of CYP3A5*3, a nonfunctional variant of CYP3A5, decreased the amount of inactive metabolite of IFO produced compared to HLMs expressing the wild type CYP3A5. The authors did not test activation IFO, but presumably activation would also be reduced with expression of this variant (*10*).

The activation of d4IFO was improved and its inactivation was reduced or completely abolished when compared to IFO for each of the polymorphic variants of CYP2B6 and CYP2C9. In general, d4IFO followed the same "patterns" for activation and inactivation by the variants as IFO. This is encouraging and supports the development of d4IFO as a potential therapeutic or as a tool for studying metabolic switching by P450s since the deuterium isotope does not appear to introduce any unexpected perturbations in metabolism by the P450s and the polymorphic variants tested.

To determine the contributions of specific P450s to IFO metabolism, and to ascertain the ability to favor activation over inactivation, we measured IFO metabolism by pooled HLMs and used chemical inhibitors to block the catalytic activities of selected P450s. The inhibition of CYP2B6 by clopidogrel inhibited both the activation and inactivation of IFO by ~40-70%. This indicates a greater contribution of CYP2B6 to the metabolism of IFO than was previously reported from studies on CYP2B6 inhibition using the monoclonal antibody inhibitor Mab2B6. Under those conditions, only an ~20% loss of activation and a 40% loss

of inactivation were observed (*12*). In a similar study with CYP2B6-genotyped HLMs, the inactivation observed with CYP2B6 inhibition was highly variable (30-90%) (*10*). The large variations in percent inhibition between these experiments is likely due to differences in the expression of P450s in the HLM (particularly CYP2B6, CYP3A4, and possibly others) samples and the polymorphic nature of the P450s. Similar results were obtained with CYP3A4 inhibition where we observed significant inhibition of both activation and inactivation of IFO (~90% for both reactions). Studies focusing only on the inactivation of IFO observed similar results with ketoconazole treatment, whereas Huang & Waxman only observed ~50% loss of both activation and inactivation in pooled HLM inhibited with the CYP3A4 inhibitor TAO (*10, 12*). Although CYP3A4 is not particularly efficient at catalyzing the inactivation of IFO (as determined in our earlier experiments), the expression of the enzyme in the liver is high. Typically CYP3A4 comprises 30-50% of all hepatic P450 enzymes (*44*). This may explain the ability of CYP3A4 to significantly contribute to the inactivation of IFO in HLM in spite of the lower catalytic efficiency for this reaction. Thus, we were unable to separate the activation and inactivation pathways with our experimental approaches, but future work will investigate other inhibitors and inhibitor concentrations to potentially optimize these studies for improved activation of IFO.

In summary, we have determined that the previously overlooked P450s CYP3A5 and CYP2C19 may play significant roles in IFO metabolism. Therefore, the potential contribution of these enzymes with regards to IFO metabolism warrants further investigation. Furthermore, the polymorphic variants of CYP2B6 and CYP2C9 exhibited significant changes in IFO metabolism, which indicates that interpatient metabolite levels may be greatly altered by the polymorphic nature of these enzymes. Additionally, our studies with

d4IFO indicate that deuteration of IFO results in an enhanced activation of IFO over inactivation for all of the P450s tested and their respective polymorphic variants suggesting that deuterium labeling of IFO can induce metabolic switching and may be of significant value in improving the therapeutic index of this widely used chemotherapeutic drug.

References

1. Zhang, J., Tian, Q., and Zhou, S. F. (2006) Clinical pharmacology of cyclophosphamide and ifosfamide, *Curr Drug Therapy 1*, 55-84.

2. Pocali, B., De Simone, M., Annunziata, M., Palmieri, S., D'Amico, M. R., Copia, C., Viola, A., Mele, G., Schiavone, E. M., and Ferrara, F. (2004) Ifosfamide, epirubicin and etoposide (IEV) regimen as salvage and mobilization therapy for refractory or early relapsing patients with aggressive non-Hodgkin's lymphoma, *Leuk Lymphoma 45*, 1605-1609.

3. Biagi, J. J., Herbert, K. E., Smith, C., Abdi, E., Leahy, M., Falkson, C., Wolf, M., Januszewicz, H., Seymour, J. F., Richards, K., Matthews, J. P., Dale, B., and Prince, H. M. (2005) A phase II study of dexamethasone, ifosfamide, cisplatin and etoposide (DICE) as salvage chemotherapy for patients with relapsed and refractory lymphoma, *Leuk Lymphoma 46*, 197-206.

4. Donfrancesco, A., Jenkner, A., Castellano, A., Ilari, I., Milano, G. M., De Sio, L., Cozza, R., Fidani, P., Deb, G., De Laurentis, C., Inserra, A., and Dominici, C. (2004) Ifosfamide/carboplatin/etoposide (ICE) as front-line, topotecan/cyclophosphamide as second-line and oral temozolomide as third-line treatment for advanced neuroblastoma over one year of age, *Acta Paediatr Suppl 93*, 6-11.

5. Buda, A., Dell'Anna, T., Signorelli, M., and Mangioni, C. (2003) Role of ifosfamide in cervical cancer: an overview, *Oncology 65 Suppl 2*, 63-66.

6. Kosmas, C., Tsavaris, N., Mylonakis, N., Tsakonas, G., Gassiamis, A., Skopelitis, H., Polyzos, A., Malamos, N., and Karabelis, A. (2007) Docetaxel-ifosfamide combination in patients with advanced breast cancer failing prior anthracycline-based regimens: results of a phase I-II study, *J Chemother 19*, 322-331.

7. Sorio, R., Lombardi, D., Spazzapan, S., La Mura, N., Tabaro, G., and Veronesi, A. (2003) Ifosfamide in advanced/disseminated breast cancer, *Oncology 65 Suppl 2*, 55-58.

8. Klastersky, J. (2003) Side effects of ifosfamide, *Oncology 65 Suppl 2*, 7-10.

9. Loebstein, R., Atanackovic, G., Bishai, R., Wolpin, J., Khattak, S., Hashemi, G., Gobrial, M., Baruchel, S., Ito, S., and Koren, G. (1999) Risk factors for long-term outcome of ifosfamide-induced nephrotoxicity in children, *J Clin Pharmacol 39*, 454-461.

10. McCune, J. S., Risler, L. J., Phillips, B. R., Thummel, K. E., Blough, D., and Shen, D. D. (2005) Contribution of CYP3A5 to hepatic and renal ifosfamide N-dechloroethylation, *Drug Metab Dispos 33*, 1074-1081.

11. Sladek, N. E. (1988) Metabolism of oxazaphosphorines, *Pharmacol Ther 37*, 301-355.

12. Huang, Z., Roy, P., and Waxman, D. J. (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide, *Biochem Pharmacol 59*, 961-972.

13. Roy, P., Yu, L. J., Crespi, C. L., and Waxman, D. J. (1999) Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles, *Drug Metab Dispos 27*, 655-666.

14. Borner, K., Kisro, J., Bruggemann, S. K., Hagenah, W., Peters, S. O., and Wagner, T. (2000) Metabolism of ifosfamide to chloroacetaldehyde contributes to antitumor activity in vivo, *Drug Metab Dispos 28*, 573-576.

15. Bruggemann, S. K., Kisro, J., and Wagner, T. (1997) Ifosfamide cytotoxicity on human tumor and renal cells: role of chloroacetaldehyde in comparison to 4-hydroxyifosfamide, *Cancer Res 57*, 2676-2680.

16. Carlson, L., Goren, M. P., Bush, D. A., Griener, J. C., Quigley, R., Tkaczewski, I., Kamen, B. A., and Weitman, S. D. (1998) Toxicity, pharmacokinetics, and in vitro hemodialysis clearance of ifosfamide and metabolites in an anephric pediatric patient with Wilms' tumor, *Cancer Chemother Pharmacol 41*, 140-146.

17. Aleksa, K., Ito, S., and Koren, G. (2004) Renal-tubule metabolism of ifosfamide to the nephrotoxic chloroacetaldehyde: pharmacokinetic modeling for estimation of intracellular levels, *J Lab Clin Med 143*, 159-162.

18. Dubourg, L., Michoudet, C., Cochat, P., and Baverel, G. (2001) Human kidney tubules detoxify chloroacetaldehyde, a presumed nephrotoxic metabolite of ifosfamide, *J Am Soc Nephrol 12*, 1615-1623.

19. Woodland, C., Ito, S., Granvil, C. P., Wainer, I. W., Klein, J., and Koren, G. (2000) Evidence of renal metabolism of ifosfamide to nephrotoxic metabolites, *Life Sci 68*, 109-117.

20. Mohrmann, M., Pauli, A., Walkenhorst, H., Schonfeld, B., and Brandis, M. (1993) Effect of ifosfamide metabolites on sodium-dependent phosphate transport in a model of proximal tubular cells (LLC-PK1) in culture, *Renal physiology and biochemistry 16*, 285-298.

21. Springate, J., Chan, K., Lu, H., Davies, S., and Taub, M. (1999) Toxicity of ifosfamide and its metabolite chloroacetaldehyde in cultured renal tubule cells, *In vitro cellular & developmental biology. Animal 35*, 314-317.

22. Kurowski, V., and Wagner, T. (1993) Comparative pharmacokinetics of ifosfamide, 4-hydroxyifosfamide, chloroacetaldehyde, and 2- and 3-dechloroethylifosfamide in patients on fractionated intravenous ifosfamide therapy, *Cancer Chemother Pharmacol 33*, 36-42.

23. Haehner, B. D., Gorski, J. C., Vandenbranden, M., Wrighton, S. A., Janardan, S. K., Watkins, P. B., and Hall, S. D. (1996) Bimodal distribution of renal cytochrome P450 3A activity in humans, *Mol Pharmacol 50*, 52-59.

24. Koch, I., Weil, R., Wolbold, R., Brockmoller, J., Hustert, E., Burk, O., Nuessler, A., Neuhaus, P., Eichelbaum, M., Zanger, U., and Wojnowski, L. (2002) Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA, *Drug Metab Dispos 30*, 1108-1114.

25. Wrighton, S. A., Schuetz, E. G., Thummel, K. E., Shen, D. D., Korzekwa, K. R., and Watkins, P. B. (2000) The human CYP3A subfamily: practical considerations, *Drug Metab Rev 32*, 339-361.

26. Ludeman, S. M., D'Alessandro, M. A., Huang, R. S., Spasojevic, I., Dolan, M. E. (2012) The use of deuterium isotope effects to shift the partitioning of cyclophosphamide and ifosfamide among competing, P450-dependent, oxidative pathways, In *American Association for Cancer Research 103rd Annual Meeting*, Cancer Research, Chicago, IL.

27. Yu, L., and Waxman, D. J. (1996) Role of cytochrome P450 in oxazaphosphorine metabolism. Deactivation via N-dechloroethylation and activation via 4-hydroxylation catalyzed by distinct subsets of rat liver cytochromes P450, *Drug Metab Dispos 24*, 1254-1262.

28. Zhang, H., Sridar, C., Kenaan, C., Amunugama, H., Ballou, D. P., and Hollenberg, P. F. (2011) Polymorphic variants of cytochrome P450 2B6 (CYP2B6.4 - CYP2B6.9) exhibit altered rates of metabolism for bupropion and efavirenz: A charge-reversal mutation in the K139E variant (CYP2B6.8) impairs formation of a functional P450-reductase complex, *J Pharmacol Exp Ther*.

29. Jenkins, C. M., Pikuleva, I., and Waterman, M. R. (1998) Expression of eukaryotic cytochromes P450 in E. coli, *Methods in molecular biology 107*, 181-193.

30. Scott, E. E., Spatzenegger, M., and Halpert, J. R. (2001) A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function, *Arch Biochem Biophys 395*, 57-68.

31. Hanna, I. H., Roberts, E. S., and Hollenberg, P. F. (1998) Molecular basis for the differences in lidocaine binding and regioselectivity of oxidation by cytochromes P450 2B1 and 2B2, *Biochemistry 37*, 311-318.

32. Domanski, T. L., Finta, C., Halpert, J. R., and Zaphiropoulos, P. G. (2001) cDNA cloning and initial characterization of CYP3A43, a novel human cytochrome P450, *Mol Pharmacol 59*, 386-392.

33. Hummel, M. A., Locuson, C. W., Gannett, P. M., Rock, D. A., Mosher, C. M., Rettie, A. E., and Tracy, T. S. (2005) CYP2C9 genotype-dependent effects on in vitro drug-

drug interactions: switching of benzbromarone effect from inhibition to activation in the CYP2C9.3 variant, *Mol Pharmacol 68*, 644-651.

34. Hustert, E., Haberl, M., Burk, O., Wolbold, R., He, Y. Q., Klein, K., Nuessler, A. C., Neuhaus, P., Klattig, J., Eiselt, R., Koch, I., Zibat, A., Brockmoller, J., Halpert, J. R., Zanger, U. M., and Wojnowski, L. (2001) The genetic determinants of the CYP3A5 polymorphism, *Pharmacogenetics 11*, 773-779.

35. Weber, G. F., and Waxman, D. J. (1993) Activation of the anti-cancer drug ifosphamide by rat liver microsomal P450 enzymes, *Biochem Pharmacol 45*, 1685-1694.

36. Highley, M. S., Schrijvers, D., Van Oosterom, A. T., Harper, P. G., Momerency, G., Van Cauwenberghe, K., Maes, R. A., De Bruijn, E. A., and Edelstein, M. B. (1997) Activated oxazaphosphorines are transported predominantly by erythrocytes, *Ann Oncol 8*, 1139-1144.

37. Boddy, A. V., Yule, S. M., Wyllie, R., Price, L., Pearson, A. D., and Idle, J. R. (1996) Intrasubject variation in children of ifosfamide pharmacokinetics and metabolism during repeated administration, *Cancer Chemother Pharmacol 38*, 147-154.

38. Davaalkham, J., Hayashida, T., Tsuchiya, K., Gatanaga, H., Nyamkhuu, D., and Oka, S. (2009) Allele and genotype frequencies of cytochrome P450 2B6 gene in a Mongolian population, *Drug Metab Dispos 37*, 1991-1993.

39. Guan, S., Huang, M., Li, X., Chen, X., Chan, E., and Zhou, S. F. (2006) Intra- and inter-ethnic differences in the allele frequencies of cytochrome P450 2B6 gene in Chinese, *Pharm Res 23*, 1983-1990.

40. Jacob, R. M., Johnstone, E. C., Neville, M. J., and Walton, R. T. (2004) Identification of CYP2B6 sequence variants by use of multiplex PCR with allele-specific genotyping, *Clin Chem 50*, 1372-1377.

41. Lang, T., Klein, K., Fischer, J., Nussler, A. K., Neuhaus, P., Hofmann, U., Eichelbaum, M., Schwab, M., and Zanger, U. M. (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver, *Pharmacogenetics 11*, 399-415.

42. Mehlotra, R. K., Ziats, M. N., Bockarie, M. J., and Zimmerman, P. A. (2006) Prevalence of CYP2B6 alleles in malaria-endemic populations of West Africa and Papua New Guinea, *Eur J Clin Pharmacol 62*, 267-275.

43. Ariyoshi, N., Ohara, M., Kaneko, M., Afuso, S., Kumamoto, T., Nakamura, H., Ishii, I., Ishikawa, T., and Kitada, M. (2011) Q172H replacement overcomes effects on the metabolism of cyclophosphamide and efavirenz caused by CYP2B6 variant with Arg262, *Drug Metab Dispos 39*, 2045-2048.

44. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians, *J Pharmacol Exp Ther 270*, 414-423.

CHAPTER 4

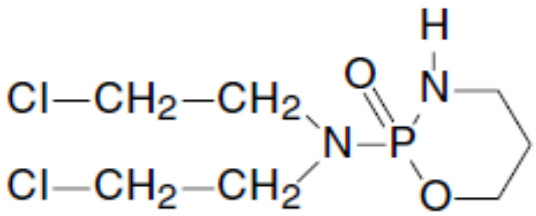
THE HYDROXYLATION AND *N*-DECHLOROETHYLATION OF DEUTERATED CYCLOPHOSPHAMIDE

Introduction

Cyclophosphamide (CPA) is a wildly used antineoplastic agent. CPA is a prodrug requiring activation via hydroxylation at the 4′ carbon. Alternatively, it can be inactivated via *N*-dechloroethylation, and this results in the production of toxic metabolites (Figure 1.6) (*1*). The *N*-dechloroethylation pathway accounts for ~10% of CPA metabolism in humans (*2-4*). CPA treatment is often associated with variable responses in patients and inconsistent exposure to the metabolites of either pathway may account for these observed interpatient discrepancies. In these studies, we focus on attempts to favor the hydroxylation, or activation, of CPA over the inactivation of CPA by introducing deuterium isotopes into the structure of CPA.

CPA was synthesized with deuterium atoms on the chloroethyl chains (Figure 4.1). It is postulated that the presence of deuterium will hinder the P450-catalyzed *N*-dechloroethylation reaction and thereby enhance the hydroxylation of the deuterium-labeled CPA (d4CPA) at the 4′ position leading to enhanced activation (*5*). Previous work using only one concentration of the drugs demonstrated increased 4′ hydroxylation of d4CPA (*5*). With CYP3A4, the fraction of the total metabolites produced from hydroxylation at the 4′ position was increased from 74% to 89% with d4CPA. With CYP3A5, this changed from 90% to 94%, and for CYP2B6, the fraction of metabolites formed due to this hydroxylation was 100% for both CPA and d4CPA (*5*). Based on these reports, we set out to more thoroughly explore the deuterium isotope effects on CPA metabolism. First, we evaluated the steady state kinetics for the metabolism of d4CPA by the wild type P450s (CYP2B6, CYP2C9, CYP2C19, and CYP3A4). The kinetic values obtained were compared with those for the metabolism of CPA by the wild type P450s. We also examined d4CPA metabolism by several of the CYP2B6 and CYP2C9 associated polymorphic variants (CYP2B6*4, CYP2B6*6-*9, and CYP2C9*2, *3). We determined that the efficiency of activation of d4CPA was relatively unchanged by most of the P450s tested, and that the efficiency of inactivation remained the same as compared to the metabolism of CPA.

CPA



d4CPA

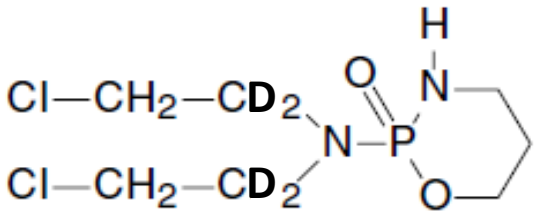


Figure 4.1 - Chemical Structures of Cyclophosphamide (CPA) and deuterium-labeled cyclophosphamide (d4CPA).

Experimental Procedures

Materials. NADPH, catalase, CPA, L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3- phosphocholine, L-α-phosphatidylserine, acrolein, CAA, 3-aminophenol, and adenosine were all purchased from Sigma Aldrich (St. Louis, MO). Pooled HLM were purchased from BD Biosciences (San Jose, CA). d4CPA was a gift from Drs. Susan Ludeman (Albany College of Pharmacy and Health Sciences) and M. Eileen Dolan (University of Chicago).

cDNA Expression and Purification of Human P450s, P450 Polymorphic Variants, and Rat Cytochrome P450 Reductase. The polymorphic variants of CYP2B6 (CYP2B6*4, CYP2B6*6– CYP2B6*9) were constructed as described by Zhang, et al. (*6*). All of the wild type and polymorphic variants were overexpressed individually in *E. coli* C41 (DE3) cells and purified as reported previously (*6*). All of the P450s were expressed as the N-terminally truncated form to increase the expression yields (*7*). Rat CPR was expressed and purified as previously (*7, 8*). CYP3A5 was a gift from Dr. James Halpert (UCSD), and CYP2C9*2 and CYP2C9*3 were gifts from Dr. Tim Tracy (University of Kentucky).

Assays for the Metabolism of CPA and d4CPA. Metabolism of CPA and d4CPA by the P450s was performed in the reconstituted system as previously described (Calinski *et al.,* in submission). The system consisted of P450 and CPR incubated at a 1:2 molar ratio for 30 min at 20°C. CYP3A4 and CYP3A5 both require a mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine (1:1:1) for function. To accommodate this, 60 μg of this lipid mixture was added to the reconstitution mixture following addition of the proteins the proteins and the system was incubated for 45 min at 4°C prior to use. The final reaction mixtures contained 0.5 μg of P450, 1 μg of CPR, 30 mM potassium phosphate (pH 7.4), 15 units of catalase, and 0-5.0

mM CPA or d4CPA in 95 µL. The reaction mixtures were incubated at 37°C for 4 min then 5 µL of NADPH (final 1 mM) was added to initiate the reaction. After 30 min at 37°C with shaking, the reactions were stopped by the addition of 40 µL of ice-cold 5.5% zinc sulfate, 40 µL of ice-cold saturated barium hydroxide, and 20 µL of ice-cold 0.01M HCl (*9, 10*). The samples were centrifuged for 10 min at 13,200 rpm and kept on ice prior to derivatization for analysis by HPLC.

Quantification of Acrolein Formation. The measurement of acrolein formation was used to quantitate the activation of CPA. In order to quantitate formation of the metabolite acrolein, 60 µL of the reaction mixture was incubated with 40 µL of the fluorescence reagent (30 mg 3-aminophenol and 30 mg hydroxylamine HCl dissolved in 5 mL 1N HCl) for 25 min at 90°C to create the fluorescent product 7-hydroxyquinoline. Using a Shimadzu HPLC system, samples were separated on an Agilent C_{18} column. The column was eluted with 20% methanol in 0.1% phosphoric acid isocratically at 0.5 mL/min. Elution of 7-hydroxyquinoline occurred at 3.2 min as monitored fluorometrically at 515 nm with an excitation of 350 nm (*9*). Calibration curves for acrolein quantification were generated by incubation of commercial acrolein in reaction mixtures containing no substrate. The standard curve was linear for all concentrations used (0-100 µM acrolein).

Quantification of Chloroacetaldehyde Formation. CAA was used to quantitate the inactivation of CPA. To detect CAA, a 60 µL aliquot of each reaction mixture was incubated with 10 µL of 100 mM adenosine in 0.25 N HCl and 10 µL of 2 M sodium acetate (pH 4.5) for 2.5 hours at 80°C. This produced the fluorescent product, 1-*N*-ethenoadenosine (*9*). The derivatized sample was resolved on an Agilent C_{18} column with a mobile phase of 20% methanol in water. The product eluted at 13.2 min and was monitored fluorometrically at

411 nm with excitation 270 nm. Calibration curves for CAA quantification were generated by incubation of commercial CAA in complete reaction mixtures containing no substrate. The standard curve was linear for CAA concentrations from 0-150 μM.

Data Analysis. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc.). The parameters for enzyme kinetics were determined using non-linear regression analysis, specifically Michaelis-Menten analysis in GraphPad Prism. Statistical significance was determined using an unpaired t-test in GraphPad Prism 6. The experiments were performed twice and the data obtained in triplicate for each point.

Results

The Metabolism of CPA and d4CPA by the Wild Type Human Cytochrome P450s. Using the reconstituted system, the steady state kinetics for the activation and inactivation of CPA and d4CPA were determined. A representative data set is shown in Figure 4.2, and the summary of all kinetic data is provided in Table 4.1. As previously noted, all of the tested P450s catalyzed the activation of CPA and CYP2B6 was the most efficient enzyme for this reaction (Calinski et al. 2013, in submission). The inactivation of CPA was catalyzed only by CYP3A4. As a result of the incorporation of the deuterium isotopes, the activation of d4CPA was inhibited for CYP2B6 and CYP2C9. The efficiency for activation of d4CPA was approximately 2-fold less than activation of CPA by these enzymes (Table 4.1). The efficiency of activation by CYP2C19 was unchanged between the drugs. Most notably, CYP3A4-mediated activation of d4CPA was 1.6-fold more efficient than activation of CPA (Figure 4.2, Table 4.1). As with CPA, only CYP3A4 catalyzed the inactivation of d4CPA. Although the inactivation of CPA and d4CPA observed with CYP3A4 did not reach a

maximum concentration of metabolite formed (i.e.- the reaction was not saturated), we have determined the kinetic values for the purpose of comparing the efficiency of CYP3A4 for inactivation of CPA and d4CPA. The K_m and V_{max} values for the inactivation of d4CPA (V_{max} = 0.3 $min^{-1}mM^{-1}$, K_m = 2.0 mM) were lower than that for CPA (V_{max} = 0.7 $min^{-1}mM^{-1}$, K_m = 3.7 mM), which ultimately resulted in a very similar efficiency for inactivation of d4CPA and CPA (Figure 4.2, Table 4.1).

The Metabolism of CPA and d4CPA by the Polymorphic Variants of CYP2B6 and CYP2C9. The polymorphic variants of CYP2B6 and CYP2C9 were examined for differences in catalytic ability with regards to CPA and d4CPA. The experiments were performed in the reconstituted system using a 1 mM concentration of drug. The concentration of drug was chosen to reflect the circulating concentration of CPA in patients (0.1 – 0.7 mM) (*11, 12*). These studies demonstrated that the polymorphic variants of all of the P450s tested exhibited slightly altered rates of metabolism for d4CPA as compared with CPA (Figure 4.3, Table 4.2). The largest variation was observed with CYP2B6*1, where activated metabolite production was 2-fold higher with CPA as opposed to d4CPA. As shown previously, CYP2B6*4 and CYP2B6*6 produced significantly less activated metabolite than the wild type P450, and CYP2B6*7 and CYP2B6*9 produced significantly more activated metabolite than the while type. Neither CYP2C9 nor its associated polymorphisms exhibited any considerable activity towards the activation of CPA or d4CPA. The inactivation data with regards to the polymorphic variants is not shown because no inactivation was observed with any of these P450s.

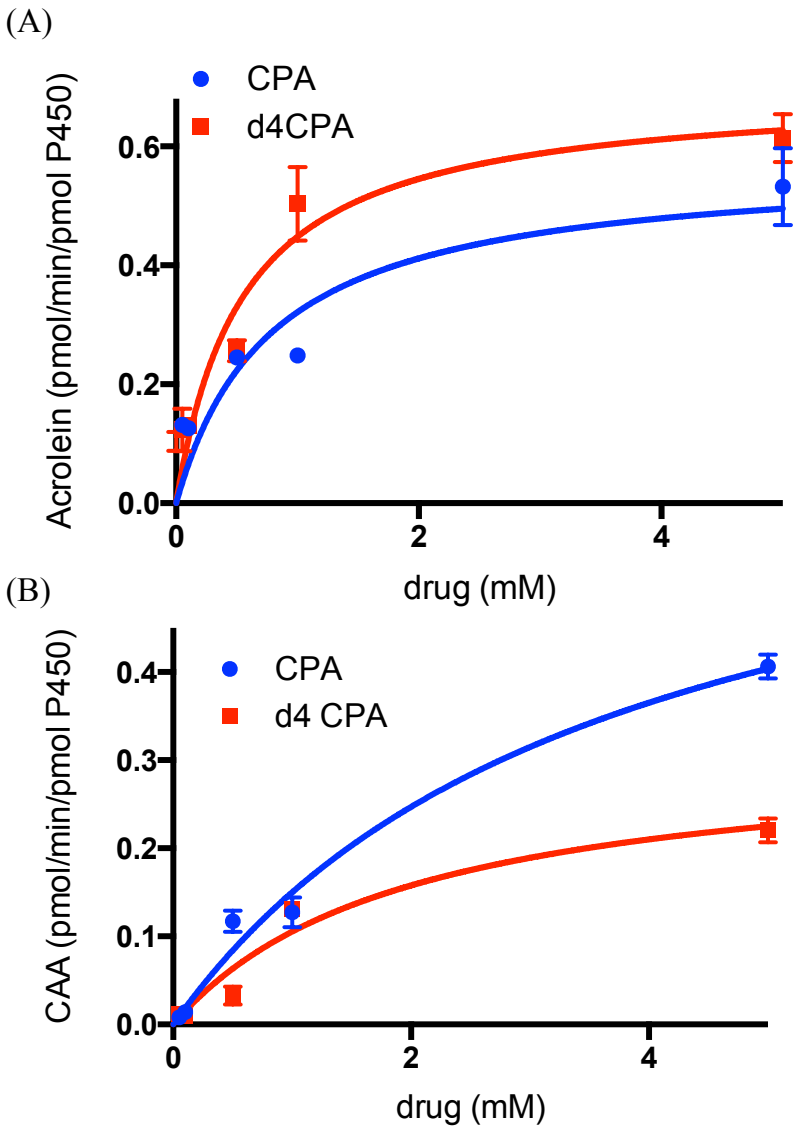


Figure 4.2 - Metabolism of CPA and d4CPA by CYP3A4. CPA (blue) or d4CPA (red) (0.1 – 5.0 mM) was incubated in the reconstituted system containing 1 μM CYP3A4 for 30 min at 37°C. Half of each sample was analyzed for the metabolite acrolein to determine the activation kinetics of CPA (A), the other half of each sample was used to measure CAA to determine the inactivation kinetics of CPA by each of the P450s (B). The details of the analytical methods are provided in the *Materials and Methods*. Shown are the nonlinear fits for the activation of the drugs determined using the Michaelis-Menten equation with Prism 6 (GraphPad Software, Inc.).

Table 4.1 - Steady State Kinetic Parameters for the Metabolism of CPA and d4CPA by the Primary P450s involved in CPA metabolism. Using Michaelis-Menten nonlinear regression (GraphPad Prism, Inc.) of the data shown in Figure 4.2, the V_{max} and K_m values for all of the P450s were determined for both activation and inactivation of CPA and d4CPA. The efficiencies of the P450s for these reactions are expressed as V_{max}/K_m.

Activation						
P450	CPA			d4CPA		
	V_{max}[a]	K_m[a]	V_{max}/K_m	V_{max}[a]	K_m[a]	V_{max}/K_m
	pmol/min/pmol P450	*mM*	*$min^{-1}mM^{-1}$*	*pmol/min/pmol P450*	*mM*	*$min^{-1}mM^{-1}$*
CYP2B6	25.2 ± 2.9	2.4 ± 0.6	10.5	9.1 ± 1.2	1.3 ± 0.4	7
CYP2C9	0.8 ± 0.1	0.4 ± 0.2	2	0.5 ± 0.1	0.5 ± 0.4	1
CYP2C19	6.4 ± 1.0	2.6 ± 1.0	2.5	6.0 ± 0.6	2.2 ± 0.5	2.8
CYP3A4	0.6 ± 0.1	0.8 ± 0.3	0.75	0.7 ± 0.1	0.6 ± 0.2	1.2
Inactivation						
P450	CPA			d4CPA		
	V_{max}[a]	K_m[a]	V_{max}/K_m	V_{max}[a]	K_m[a]	V_{max}/K_m
	pmol/min/pmol P450	*mM*	*$min^{-1}mM^{-1}$*	*pmol/min/pmol P450*	*mM*	*$min^{-1}mM^{-1}$*
CYP2B6	no CAA formed		0	no CAA formed		0
CYP2C9	no CAA formed		0	no CAA formed		0
CYP2C19	no CAA formed		0	no CAA formed		0
CYP3A4	0.7 ± 0.1	3.7 ± 0.9	0.19	0.3 ± 0.04	2.0 ± 0.6	0.15

[a] Mean ± S.E.M.

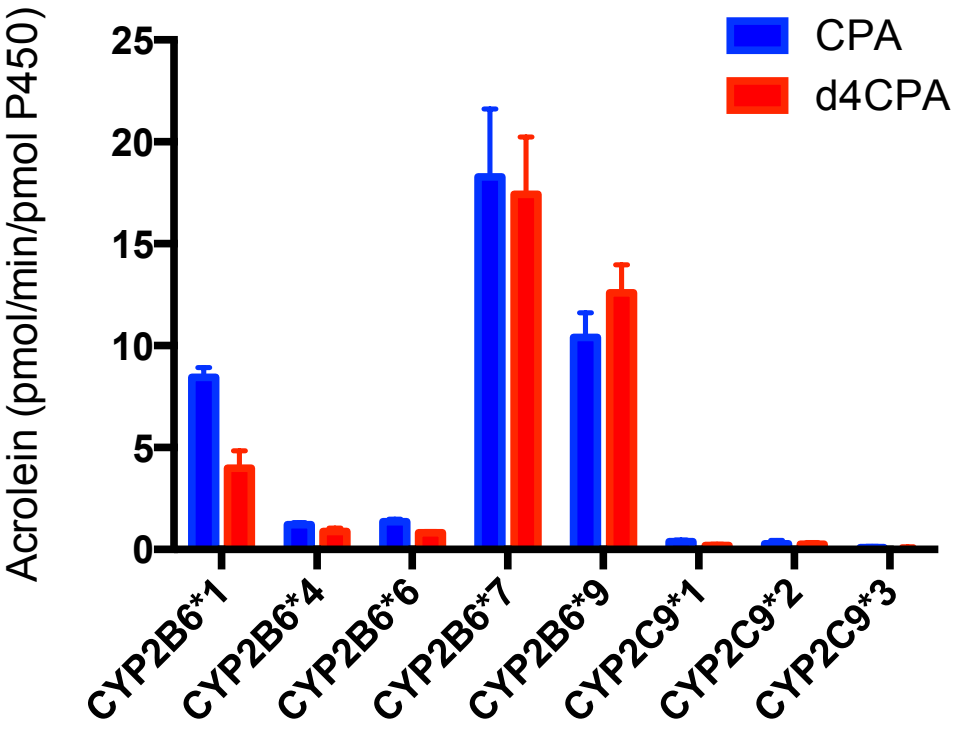


Figure 4.3 - Activation of CPA and d4CPA by the Polymorphic Variants of CYP2B6 and CYP2C9. CPA (blue) or d4CPA (red) (1 mM) was incubated in the reconstituted system with 1 µM of P450 for 30 min at 37°C. Half of each sample was analyzed for the metabolite acrolein to determine the rate of activation of CPA for each of the polymorphic variants. The other half of each sample was used to detect CAA to determine the rate of inactivation of IFO for each of the polymorphic variants, the data are not shown because no CAA could be detected. The details of the analytical methods are provided in the *Materials and Methods*. The average for each experimental condition that was performed in triplicate is shown. Statistical significance was assessed using an unpaired t-test in Prism 6 (GraphPad Software, Inc.). It was determined that the acrolein produced from d4CPA not not differ significantly from acrolein produced from CPA for any of the polymorphic variants.

Table 4.2 - The Activation of CPA and d4CPA by the Polymorphic Variants of CYP2B6 and CYP2C9. The variants were assessed for changes in activation and inactivation of CPA and d4CPA at 1 mM drug. The data from Figure 4.3 are expressed as pmol/min/pmol P450 of acrolein produced, and fold change from wild type. The ratio of activated CPA to activated d4CPA was also calculated for each of the polymorphic variants. The inactivation data are not shown because neither CPA nor d4CPA was inactivated by any of the polymorphic variants.

	Activation				
P450	CPA		d4CPA		Ratio of Activation of CPA to d4CPA
	Acrolein Formed[a]	Percent Change over Wild Type	Acrolein Formed[a]	Percent Change over Wild Type	
	pmol/min/pmol P450	*%*	*pmol/min/pmol P450*	%	
CYP2B6*1	8.4 ± 0.5	0.0	4.0 ± 0.9	0.0	2.1
CYP2B6*4	1.2 ± 0.1	-85.7	0.9 ± 0.2	-77.5	1.3
CYP2B6*6	1.4 ± 0.1	-83.3	0.8 ± 0.1	-80.0	1.8
CYP2B6*7	18.3 ± 3.3	117.9	17.4 ± 2.8	335.0	1.1
CYP2B6*9	10.4 ± 1.2	23.8	12.6 ± 1.4	215.0	0.83
CYP2C9*1	0.4 ± 0.1	0.0	0.2 ± 0.05	0.0	2.0
CYP2C9*2	0.3 ± 0.2	-25.0	0.3 ± 0.1	50.0	1.0
CYP2C9*3	0.2 ± 0.1	-50.0	0.01 ± 0.1	-95.0	20.0

[a] Mean ± S.E.M.

Discussion

We have investigated the metabolic effects of introducing deuterium isotopes to the cancer chemotherapeutic CPA. To do this, we used purified P450s in the reconstituted system and compared both the activation and inactivation pathways for d4CPA to those for CPA. We determined that both drugs have relatively similar rates of metabolism and that the presence of deuterium in d4CPA appears to result in reduced activation by many of the P450s.

Previously, d4CPA was analyzed for changes in its activation and inactivation pathways by CYP2B6, CYP3A4, and CYP3A5 at a concentration of 1 mM (*5*). It was determined that the fraction of the hydroxylated products was slightly enhanced by introduction of the deuterium isotope. For instance, with CYP3A4, 89% of the metabolites formed from d4CPA were the result of hydroxylation versus 74% for CPA. The other P450s tested all exhibited much smaller changes in the formation of the activated product (*5*). Based on those studies, we examined the steady state kinetics to more thoroughly investigate d4CPA as a potential improvement to CPA. Only CYP3A4 exhibited enhanced metabolism of d4CPA as compared with metabolism of CPA. The activation of d4CPA by CYP3A4 was enhanced almost 2-fold (V_{max}/K_m = 0.75 $min^{-1}mM^{-1}$ for CPA and V_{max}/K_m = 1.2 $min^{-1}mM^{-1}$ for d4CPA), while the inactivation of d4CPA by CYP3A4 was similar to the inactivation of CPA (V_{max}/K_m = 0.19 $min^{-1}mM^{-1}$ for CPA and V_{max}/K_m = 0.15 $min^{-1}mM^{-1}$ for d4CPA). Therefore, the overall result is a mild metabolic switch to favor hydroxylation of d4CPA by CYP3A4. Interestingly, as we tested activation and inactivation at 1 mM, we can directly compare our results with results obtained by Ludeman, et al. (*5*). For CYP3A4, the amount of activated metabolite produced from 1 mM CPA was 97% of the total metabolites

produced, which was reduced to 85% for d4CPA. These variations can be the result of differences in the experimental set up and the assays used to measure metabolite production. Therefore, it is best to compare the metabolism of d4CPA and CPA within the same system as we have done. The inactivation of d4CPA did not differ from that of CPA. Thus, the inclusion of deuterium did enhance hydroxylation but did not reduce *N*-dechloroethylation.

The polymorphic variants of CYP2B6 and CYP2C9 were evaluated for catalytic differences with regards to activation and inactivation of d4CPA. The amount of active metabolite produced by each of the variants was slightly reduced compared to amount of activated metabolite produced from CPA, with the exception of CYP2B6*9 which had a slight increase in active metabolite from d4CPA. None of these changes were statistically significant. None of the polymorphic variants of CYP2B6 or CYP2C9 catalyzed the inactivation of d4CPA or CPA. It is encouraging that no large discrepancies in the activation or inactivation of the drugs were observed, because this indicates that the drugs and the enzymes are largely not perturbed by the inclusion of the deuterium isotopes.

In summary, the inclusion of deuterium isotopes on the chloroethyl chains of CPA did not reduce the inactivation of CPA, only slightly enhanced the activation of CPA for CYP3A4, and actually hindered the activation of CPA for CYP2B6 and CYP2C9 (Figure 4.2 and Table 4.1). Our work demonstrates that deuterium labeling is the not a viable option for the enhancement of CPA metabolism. As mentioned previously, *N*-dechloroethylation only accounts for ~10% of CPA metabolism and therefore inhibiting this pathway may not affect patient outcome as greatly as hoped. The inhibition of *N*-dechloroethylation may be more important for the related drug, ifosfamide, as 25-60% of this drug is *N*-dechloroethylated by the P450s (*2-4*). Deuterium labeling of IFO has proven more effective at both inhibiting

inactivation and enhancing activation. Therefore efforts towards the development of d4IFO may be more rewarding (Calinski et al. 2013, in preparation).

References

1. Sladek, N. E. (1988) Metabolism of oxazaphosphorines, *Pharmacol Ther 37*, 301-355.

2. Carlson, L., Goren, M. P., Bush, D. A., Griener, J. C., Quigley, R., Tkaczewski, I., Kamen, B. A., and Weitman, S. D. (1998) Toxicity, pharmacokinetics, and in vitro hemodialysis clearance of ifosfamide and metabolites in an anephric pediatric patient with Wilms' tumor, *Cancer Chemother Pharmacol 41*, 140-146.

3. Bruggemann, S. K., Kisro, J., and Wagner, T. (1997) Ifosfamide cytotoxicity on human tumor and renal cells: role of chloroacetaldehyde in comparison to 4-hydroxyifosfamide, *Cancer Res 57*, 2676-2680.

4. Borner, K., Kisro, J., Bruggemann, S. K., Hagenah, W., Peters, S. O., and Wagner, T. (2000) Metabolism of ifosfamide to chloroacetaldehyde contributes to antitumor activity in vivo, *Drug Metab Dispos 28*, 573-576.

5. Ludeman, S. M., D'Alessandro, M. A., Huang, R. S., Spasojevic, I., Dolan, M. E. (2012) The use of deuterium isotope effects to shift the partitioning of cyclophosphamide and ifosfamide among competing, P450-dependent, oxidative pathways, In *American Association for Cancer Research 103rd Annual Meeting*, Cancer Research, Chicago, IL.

6. Zhang, H., Sridar, C., Kenaan, C., Amunugama, H., Ballou, D. P., and Hollenberg, P. F. (2011) Polymorphic variants of cytochrome P450 2B6 (CYP2B6.4 - CYP2B6.9) exhibit altered rates of metabolism for bupropion and efavirenz: A charge-reversal mutation in the K139E variant (CYP2B6.8) impairs formation of a functional P450-reductase complex, *J Pharmacol Exp Ther*.

7. Hanna, I. H., Reed, J. R., Guengerich, F. P., and Hollenberg, P. F. (2000) Expression of human cytochrome P450 2B6 in Escherichia coli: characterization of catalytic activity and expression levels in human liver, *Arch Biochem Biophys 376*, 206-216.

8. Scott, E. E., Spatzenegger, M., and Halpert, J. R. (2001) A truncation of 2B subfamily cytochromes P450 yields increased expression levels, increased solubility, and decreased aggregation while retaining function, *Arch Biochem Biophys 395*, 57-68.

9. Weber, G. F., and Waxman, D. J. (1993) Activation of the anti-cancer drug ifosphamide by rat liver microsomal P450 enzymes, *Biochem Pharmacol 45*, 1685-1694.

10. Chang, T. K., Weber, G. F., Crespi, C. L., and Waxman, D. J. (1993) Differential activation of cyclophosphamide and ifosphamide by cytochromes P-450 2B and 3A in human liver microsomes, *Cancer Res 53*, 5629-5637.

11. Chen, T. L., Passos-Coelho, J. L., Noe, D. A., Kennedy, M. J., Black, K. C., Colvin, O. M., and Grochow, L. B. (1995) Nonlinear pharmacokinetics of cyclophosphamide

in patients with metastatic breast cancer receiving high-dose chemotherapy followed by autologous bone marrow transplantation, *Cancer Res 55*, 810-816.

12. Ayash, L. J., Wright, J. E., Tretyakov, O., Gonin, R., Elias, A., Wheeler, C., Eder, J. P., Rosowsky, A., Antman, K., and Frei, E., 3rd. (1992) Cyclophosphamide pharmacokinetics: correlation with cardiac toxicity and tumor response, *J Clin Oncol 10*, 995-1000.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The oxazaphosphorines, cyclophosphamide (CPA) and ifosfamide (IFO), are two DNA-alkylating agents that are widely used in cancer chemotherapy. Although the drugs have contributed to the effective treatment of thousands of patients over the course of decades, variable patient responses and severe adverse drug reactions persist and limit the use of these drugs' (*1*). The toxicities and variations in patient response are related to the interindividual variations in the metabolism of the oxazaphosphorines. Both compounds are administered as prodrugs and require bioactivation by the cytochrome P450s (P450s) via 4-hydroxylation. After hydroxylation, the drugs undergo a series of spontaneous reactions to produce the therapeutically active nitrogen mustard, which can then alkylate DNA and ultimately initiate apoptosis. An alternative route of metabolism involves the P450-catalyzed *N*-dechloroethylation of the drugs. This reaction results in inactivation of the drugs, as well as the production of the therapeutically inactive yet toxic metabolites: dechloroethylated-CPA or dechloroethylated-IFO and chloroacetaldehyde (CAA) (*2*). The primary P450s

responsible for these reactions differ between CPA and IFO, and these P450s are highly polymorphic, which can influence the catalytic ability of the enzymes (*3-5*). In particular, CYP2B6 has over 28 known allelic variants, some of which are expressed at upwards of 30% in some populations (*6-8*). A full understanding of the factors affecting the pharmacokinetics and pharmacogenomics of CPA and IFO is important to optimize the treatment regimens utilizing CPA and IFO in cancer chemotherapies. The work in this thesis has focused on advancing our basic understanding of hydroxylation and *N*-dechloroethylation of CPA and IFO by the human P450s and their primary polymorphisms. We have also sought to investigate the potential for beneficially manipulating these two pathways using P450 inhibitors and chemical modifications of the drugs.

To investigate the metabolism of CPA, experiments were performed using purified enzymes in a recombinant system. Utilizing the recombinant system allowed us to individually assess the P450s for their involvement in both pathways, regardless of the expression levels of the protein and other complicating factors present in more complex systems. Our investigations confirmed that CYP2B6 is the most efficient enzyme for the hydroxylation, or activation, of CPA (*4, 5*). In the same system, several polymorphic variants of CYP2B6 (CYP2B6*4-*9) were analyzed for their ability to catalyze hydroxylation of CPA. This was the most extensive undertaking of its kind to date. Our studies indicate that all of the polymorphic variants tested have decreased abilities to activate CPA, when compared to wild type. Given that most polymorphisms have lowered expression levels *in vivo,* the combination of these factors may be detrimental for patients (*9-11*). Yet, multiple studies have failed to find any correlation between CYP2B6 genotype and therapeutic outcome of CPA (*12, 13*). The lack of genotype effect on the pharmacokinetics

in patients is likely due to the contribution of other P450s with regards to the therapeutic activation of CPA. In accordance with this hypothesis, our studies show that CYP2C19, CYP3A4, CYP3A5, and CYP2C9 also catalyze the activation of CPA, but with less efficiency than CYP2B6 (listed above in rank order of efficiency). The relevance of these enzymes for the *in vivo* metabolism of CPA will depend on the route of administration (since P450 expression varies in organs), expression of various P450 polymorphic variants, and co-administration of potential P450 inhibitors that may be included in treatment regimens or for other existing conditions in patients. Encouragingly, only CYP3A4 inactivated CPA, which led us to question whether we could inhibit CPA inactivation and production of toxic metabolites by inhibiting CYP3A4 activity. To test this, we used ketoconazole to inhibit CYP3A4 in human liver microsomes (HLM). The inhibition of CYP3A4 resulted in a complete loss of inactivation of CPA, but also reduced the activation by ~50%. Thus, in spite of the low catalytic efficiency for CPA activation associated with CYP3A4, it is clear that this enzyme is important for this reaction in HLM, likely due to the high levels of expression of the enzyme in the liver. Future work will focus on attempting to separate the two pathways in HLM with different CYP3A4 inhibitors or using various concentrations of ketoconazole. Additionally, studies with other tissues, such as tumors, would illuminate the importance of P450s in the target tissues which may be of interest for CPA activation and may alter the P450s we deem to be important for CPA metabolism in the clinical setting.

IFO has proven to be more effective than CPA in a wide range of malignant diseases (*14-19*). Yet, IFO use is limited owing to the severe toxicities associated with its use. IFO-induced toxicities include nephrotoxicity, which occurs in ~30% of the patient population, and neurotoxicities, which occur in ~20% of the patient population (*20-22*). IFO-associated

toxicities are directly related to the metabolism of the drug. In humans it is estimated that 25-60% of IFO is *N*-dechloroethylated to give CAA whereas only 10% of CPA undergoes this reaction (*23-25*). It is suggested that elevated levels of CAA in IFO-treated patients is responsible for the development of the IFO-specific nephro- and neurotoxicities. Therefore, as with CPA, we investigated the P450-catalyzed metabolism of IFO, as well as the potential to control the metabolism to favor hydroxylation. These studies demonstrated that CYP3A5 and CYP2C19 were the most efficient enzymes for catalyzing IFO activation, and importantly that CYP2C19 did not catalyze the *N*-dechloroethylation of IFO. The role of CYP2C19 in IFO metabolism has not been previously studied, although it's significance may not be surprising, given that CYP2C19 is important for the metabolism of CPA. CYP2C19 may prove to be the ideal P450 to target for IFO metabolism, as the efficiency for activation is high, and inactivation was not observed. Inactivation of IFO by CYP3A5 has been implicated in the development of nephrotoxicity in patients because CYP3A5 expression is high in the kidney and therefore, CAA formation may be concentrated at that site. Indeed, human kidney microsomes that express the nonfunctional polymorphic variant CYP3A5*3, had less CAA production than human kidney microsomes with wild type protein (*20*). It would be interesting to investigate genotyped IFO-treated patients to determine if there is a genotype-phenotype correlation between CYP3A5 and the development of nephrotoxicities. Our data supports that CYP3A5 may play a role in this organ-specific toxicity; however, the most efficient P450 for IFO inactivation is CYP2B6 and therefore, future studies should also focus on CYP2B6 expression in the kidney. The polymorphic variants of CYP2B6 were also tested for differences in the catalytic abilities for activation and inactivation of IFO. Only slight differences were observed for either reaction for all of the polymorphisms tested. The

ability to favor hydroxylation of IFO over *N*-dechloroethylation was assessed in two ways for IFO. First we tested P450 inhibition in HLM. With P450 inhibition, we were unable to separate activation and inactivation of IFO. This agrees with our earlier studies where it was determined that both reactions could be catalyzed by multiple P450s and therefore separation of the pathways using P450 inhibition in a complex system would be difficult. In the second set of studies aimed at favoring hydroxylation of the drug, IFO was synthesized with deuterium on the chloroethyl chains and both metabolic pathways were examined for all of the P450s tested previously. The placement of deuterium was intended to hinder the dechloroethylation and thereby enhance hydroxylation of IFO (*26*). Indeed, we observed a slight favoring of hydroxylation over *N*-dechloroethylation but it was not statistically significant. Future work will continue to explore labeled IFO (d4IFO) as a potential therapeutic. Studies will include analyzing the DNA alkylating properties of d4IFO and *in vivo* investigations.

We also assessed the metabolism of deuterium labeled CPA (d4CPA) by the P450s. As with d4IFO, our goal was to see if it might be possible to eliminate or reduce *N*-dechloroethylation of CPA and thereby enhance hydroxylation (*26*). As mentioned previously, only 10% CPA undergoes *N*-dechloroethylation and therefore toxicities associated with inactivation of the drug are less problematic than with IFO (*23-25*). However, the large interpatient variabilities associated with CPA may be better controlled with tighter regulation of the two metabolic pathways. We determined that the incorporation of deuterium in the chloroethyl chains of CPA did not significantly alter the *N*-dechloroethylation of the drug. As with CPA, only CYP3A4 catalyzed this reaction and it was equally efficient for d4CPA and CPA. Additionally, the hydroxylation of d4CPA by

CYP2B6 was greatly inhibited, as compared to CPA hydroxylation. Therefore, we concluded that d4CPA would not be a better alternative to CPA.

Taken together, the work compiled in this thesis has enhanced our mechanistic understanding of the metabolism of the two oxazaphosphorines. It is our hope that this information will encourage future investigations focused on the improvement of current cancer chemotherapies, drug design, and the use of pharmacogenomics to design enhanced chemotherapeutic regimens.

References

1. Zhang, J., Tian, Q., and Zhou, S. F. (2006) Clinical pharmacology of cyclophosphamide and ifosfamide, *Curr Drug Therapy 1*, 55-84.

2. Sladek, N. E. (1988) Metabolism of oxazaphosphorines, *Pharmacol Ther 37*, 301-355.

3. Yu, L., and Waxman, D. J. (1996) Role of cytochrome P450 in oxazaphosphorine metabolism. Deactivation via N-dechloroethylation and activation via 4-hydroxylation catalyzed by distinct subsets of rat liver cytochromes P450, *Drug Metab Dispos 24*, 1254-1262.

4. Roy, P., and Waxman, D. J. (2006) Activation of oxazaphosphorines by cytochrome P450: application to gene-directed enzyme prodrug therapy for cancer, *Toxicol In Vitro 20*, 176-186.

5. Huang, Z., Roy, P., and Waxman, D. J. (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide, *Biochem Pharmacol 59*, 961-972.

6. Lang, T., Klein, K., Fischer, J., Nussler, A. K., Neuhaus, P., Hofmann, U., Eichelbaum, M., Schwab, M., and Zanger, U. M. (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver, *Pharmacogenetics 11*, 399-415.

7. Mehlotra, R. K., Ziats, M. N., Bockarie, M. J., and Zimmerman, P. A. (2006) Prevalence of CYP2B6 alleles in malaria-endemic populations of West Africa and Papua New Guinea, *Eur J Clin Pharmacol 62*, 267-275.

8. Davaalkham, J., Hayashida, T., Tsuchiya, K., Gatanaga, H., Nyamkhuu, D., and Oka, S. (2009) Allele and genotype frequencies of cytochrome P450 2B6 gene in a Mongolian population, *Drug Metab Dispos 37*, 1991-1993.

9. Ekins, S., Vandenbranden, M., Ring, B. J., Gillespie, J. S., Yang, T. J., Gelboin, H. V., and Wrighton, S. A. (1998) Further characterization of the expression in liver and catalytic activity of CYP2B6, *J Pharmacol Exp Ther 286*, 1253-1259.

10. Faucette, S. R., Hawke, R. L., Lecluyse, E. L., Shord, S. S., Yan, B., Laethem, R. M., and Lindley, C. M. (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity, *Drug Metab Dispos 28*, 1222-1230.

11. Lang, T., Klein, K., Richter, T., Zibat, A., Kerb, R., Eichelbaum, M., Schwab, M., and Zanger, U. M. (2004) Multiple novel nonsynonymous CYP2B6 gene polymorphisms in Caucasians: demonstration of phenotypic null alleles, *J Pharmacol Exp Ther 311*, 34-43.

12. Timm, R., Kaiser, R., Lotsch, J., Heider, U., Sezer, O., Weisz, K., Montemurro, M., Roots, I., and Cascorbi, I. (2005) Association of cyclophosphamide pharmacokinetics to polymorphic cytochrome P450 2C19, *Pharmacogenomics J 5*, 365-373.

13. Ekhart, C., Doodeman, V. D., Rodenhuis, S., Smits, P. H., Beijnen, J. H., and Huitema, A. D. (2008) Influence of polymorphisms of drug metabolizing enzymes (CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, GSTA1, GSTP1, ALDH1A1 and ALDH3A1) on the pharmacokinetics of cyclophosphamide and 4-hydroxycyclophosphamide, *Pharmacogenet Genomics 18*, 515-523.

14. Buda, A., Dell'Anna, T., Signorelli, M., and Mangioni, C. (2003) Role of ifosfamide in cervical cancer: an overview, *Oncology 65 Suppl 2*, 63-66.

15. Sorio, R., Lombardi, D., Spazzapan, S., La Mura, N., Tabaro, G., and Veronesi, A. (2003) Ifosfamide in advanced/disseminated breast cancer, *Oncology 65 Suppl 2*, 55-58.

16. Pocali, B., De Simone, M., Annunziata, M., Palmieri, S., D'Amico, M. R., Copia, C., Viola, A., Mele, G., Schiavone, E. M., and Ferrara, F. (2004) Ifosfamide, epirubicin and etoposide (IEV) regimen as salvage and mobilization therapy for refractory or early relapsing patients with aggressive non-Hodgkin's lymphoma, *Leuk Lymphoma 45*, 1605-1609.

17. Kosmas, C., Tsavaris, N., Mylonakis, N., Tsakonas, G., Gassiamis, A., Skopelitis, H., Polyzos, A., Malamos, N., and Karabelis, A. (2007) Docetaxel-ifosfamide combination in patients with advanced breast cancer failing prior anthracycline-based regimens: results of a phase I-II study, *J Chemother 19*, 322-331.

18. Donfrancesco, A., Jenkner, A., Castellano, A., Ilari, I., Milano, G. M., De Sio, L., Cozza, R., Fidani, P., Deb, G., De Laurentis, C., Inserra, A., and Dominici, C. (2004) Ifosfamide/carboplatin/etoposide (ICE) as front-line, topotecan/cyclophosphamide as second-line and oral temozolomide as third-line treatment for advanced neuroblastoma over one year of age, *Acta Paediatr Suppl 93*, 6-11.

19. Biagi, J. J., Herbert, K. E., Smith, C., Abdi, E., Leahy, M., Falkson, C., Wolf, M., Januszewicz, H., Seymour, J. F., Richards, K., Matthews, J. P., Dale, B., and Prince, H. M. (2005) A phase II study of dexamethasone, ifosfamide, cisplatin and etoposide (DICE) as salvage chemotherapy for patients with relapsed and refractory lymphoma, *Leuk Lymphoma 46*, 197-206.

20. McCune, J. S., Risler, L. J., Phillips, B. R., Thummel, K. E., Blough, D., and Shen, D. D. (2005) Contribution of CYP3A5 to hepatic and renal ifosfamide N-dechloroethylation, *Drug Metab Dispos 33*, 1074-1081.

21. Loebstein, R., Atanackovic, G., Bishai, R., Wolpin, J., Khattak, S., Hashemi, G., Gobrial, M., Baruchel, S., Ito, S., and Koren, G. (1999) Risk factors for long-term

outcome of ifosfamide-induced nephrotoxicity in children, *J Clin Pharmacol 39*, 454-461.

22. Klastersky, J. (2003) Side effects of ifosfamide, *Oncology 65 Suppl 2*, 7-10.

23. Carlson, L., Goren, M. P., Bush, D. A., Griener, J. C., Quigley, R., Tkaczewski, I., Kamen, B. A., and Weitman, S. D. (1998) Toxicity, pharmacokinetics, and in vitro hemodialysis clearance of ifosfamide and metabolites in an anephric pediatric patient with Wilms' tumor, *Cancer Chemother Pharmacol 41*, 140-146.

24. Bruggemann, S. K., Kisro, J., and Wagner, T. (1997) Ifosfamide cytotoxicity on human tumor and renal cells: role of chloroacetaldehyde in comparison to 4-hydroxyifosfamide, *Cancer Res 57*, 2676-2680.

25. Borner, K., Kisro, J., Bruggemann, S. K., Hagenah, W., Peters, S. O., and Wagner, T. (2000) Metabolism of ifosfamide to chloroacetaldehyde contributes to antitumor activity in vivo, *Drug Metab Dispos 28*, 573-576.

26. Ludeman, S. M., D'Alessandro, M. A., Huang, R. S., Spasojevic, I., Dolan, M. E. (2012) The use of deuterium isotope effects to shift the partitioning of cyclophosphamide and ifosfamide among competing, P450-dependent, oxidative pathways, In *American Association for Cancer Research 103rd Annual Meeting*, Cancer Research, Chicago, IL.