ESTABLISHING METRONIDAZOLE AS A NOVEL BIOMARKER OF CYP2A6 ACTIVITY

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OF CYP2A6 ACTIVITY

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University of Missouri-Kansas City, 2018

ABSTRACT

CYP2A6 is a polymorphically expressed enzyme with variation associated with smoking behavior, cessation success, lung cancer risk, and differential drug exposure to numerous medications including anti-infectious and chemotherapeutic agents. Current CYP2A6 probes that could provide insight *a priori* into variability are limited due to safety, accessibility, general applicability and/or enzyme specificity. Therefore, the purpose of this research was to develop a novel biomarker to understand variability in CYP2A6 activity in humans. A step-wise approach was utilized and included evaluation of the ability of metronidazole and nicotine (current gold standard) to modulate CYP2A6 activity and expression*in vitro*, validation of a novel analytical method to determine the concentrations of metronidazole and 2-hydroxymetronidazole in human plasma, and finally, comparison of metronidazole to nicotine (via metabolite/parent ratio) for use as a CYP2A6 phenotyping probe in humans. Metronidazole and nicotine had minimal effects on CYP2A6 expression or activity *in vitro* at therapeutically relevant concentrations and are predicted to not lead to meaningful clinical impact. Using a low volume of human plasma (10 μL), metronidazole and 2-hydroxymetronidazole were simultaneously quantitated by a novel, validated UPLC-MS/MS method that is among the most sensitive to date. The metronidazole probe measure

(2-hydroxymetronidazole/metronidazole ratio in plasma) proved well-tolerated, highly specific for CYP2A6, and robust with a wide window of use. This novel probe measure was also able to dichotomize individuals based on genotype-predicted phenotype in a way that mirrored the nicotine probe measures. In summary, this work establishes the metronidazole probe measure as a novel biomarker of CYP2A6 variability in humans, thus providing a tool to understand human diversity and potentially, improve health outcomes in a diverse pool of individuals. Future studies evaluating the use of metronidazole as a probe of CYP2A6 activity in sub-populations of humans would be useful to further investigate the performance of this tool in special populations at risk for poor health outcomes.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined a dissertation titled, "Establishing Metronidazole as a Novel Biomarker of CYP2A6 Activity," presented by Stephani L. Stancil, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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DEDICATION

To Evelyn Lili Stancil, my little scientist. May you never cease asking questions, being curious, and challenging the status quo with love and wisdom beyond your years. You inspire me.

To Quinton Walter Almai Stancil, my focused observer. May you pursue your passions with your great energy, light, and love and continue making the world brighter with your smile. Your sweet encouragement is such a gift.

CHAPTER 1

INTRODUCTION

Phenotyping Probes as Biomarkers

An adage attributed to the 16th century scientist, Paracelsus, states that the dose makes the poison (Borzelleca, 2000). This describes the belief that all things have the potential to be toxic if they are able to overwhelm a certain biological system. One of the greatest examples is the toxicity of medications used to treat human disease. Human diversity is an important ingredient in the recipe for drug toxicity. Still often the standard of care –applying the same dose to all patients – can potentially serve as a catalyst for this outcome. Understanding elements of biological diversity contributing to variability in drug response has become a target of modern medicine.

The Precision Medicine Initiative, launched in 2016, is a large-scale project administered in part by the National Institutes of Health and focused on understanding how factors affecting the individual can be used to inform prevention and/or treatment regimens (Reference, n.d.). When looking specifically at drug therapy, the pursuit of precision medicine has led to the development and implementation of tools to create a world where the uniqueness of the individual patient can be identified and used to craft the best drug treatment targeted directly at that human. Biomarkers are examples of tools able to assist in providing information *a priori* regarding diversity. A biomarker is defined by the Institutes of Medicine as "a characteristic that is objectively measured…as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001, p. 91). Biomarkers can be used to help make decisions about drug therapy and predict adverse events associated with

variable exposure. The benefits of such use can be realized in the reduction of treatment costs and elevation of drug safety (Institute of Medicine (US) Forum on Drug Discovery, Development, and Translation, 2009).

Phenotyping probes are one example of such biomarkers. They are used to understand the physical function (i.e., phenotype) of a particular enzyme *in vitro* or *in vivo*. The information gained can be used to predict variability in exposure to the particular drug and to potentially correlate activity to health outcomes. In the context of drug metabolism, a drug (i.e., probe) is administered, and the product of biotransformation is measured. Often metabolite/parent ratios serve as the biomarker of the activity of the particular metabolic pathway. Phenotyping probes have been widely studied in the large superfamily of drug metabolism enzymes in the liver, the cytochrome P450 (CYP) enzymes.

The CYP superfamily includes 57 human enzymes and is responsible for 80% of oxidative phase I metabolism, clearing 65-70% of drugs from the body (Zhou, Ingelman-Sundberg, & Lauschke, 2017). Differences in the activity of these pathways may yield variability in exposure to active compounds. Variability in exposure may yield differential response to therapeutic drugs leading to treatment failure and/or adverse reactions. Variability may be influenced by genetic polymorphisms, environmental exposures, concurrent medications, or endogenous compounds. Measuring activity with phenotyping probes provides a summary of the many factors at play rather than a snapshot of one potential factor, such as when obtaining genotype alone. Specific examples of phenotyping probes used to determine the activity of certain CYP enzymes include bupropion (CYP2B6), phenytoin (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A4) (Streetman, Bertino, & Nafziger, 2000).

Important considerations when selecting a probe drug, or substrate, include specificity (for the reaction measured), safety, tolerability, cost, and availability of the compound. It is also important to understand the effect the probe drug may have, such as inhibition or induction, on the pathway of interest. Similarly, the probe itself should reflect any such effects (i.e., inhibition or induction) that occur in the scenario of interest. *In vitro* studies often shed light on these characteristics of the potential probe drug (Faber, Jetter, & Fuhr, 2005). *In vivo* studies can then be useful to characterize the performance of the probe measure to accurately reflect the activity of the enzyme within the complexity of the human system.

Statement of Purpose

The purpose of this work is to validate metronidazole as a novel, safe phenotyping probe in humans to understand variability in CYP2A6. To do so requires the progression of work starting first with *in vitro* experiments that examine the effects of metronidazole and nicotine on CYP2A6 (i.e., its ability to induce or inhibit) followed by evaluation of the performance of metronidazole metabolite ratio *in vivo* as a probe against the current "gold standard" nicotine metabolite ratio. The following specific aims have been set forth to accomplish this goal:

Specific Aim 1: To investigate the inhibition effect, if any, of nicotine and metronidazole on CYP2A6 in pooled human liver microsomes (Chapter 2)

Specific Aim 2: To evaluate the induction effect, if any, of nicotine and metronidazole on CYP2A6 in primary human hepatocytes (Chapter 2)

Specific Aim 3: To validate the conversion of metronidazole to 2-OH metronidazole as an i*n vivo* CYP2A6 phenotyping probe.

3a: Validate an UPLC-MS/MS method for detection of metronidazole and 2-hydroxymetronidazole in human plasma (Chapter 3)

3b: Correlate plasma metabolite/parent ratio of metronidazole to the "gold standard" nicotine to determine validity of metronidazole as novel CYP2A6 phenotyping probe in humans (Chapter 4)

3c: Explore the frequency and subsequent effects, if any, of CYP2A6 allelic variants present in the cohort of subjects on the biotransformation of metronidazole in a heterogeneous, Midwestern population (Chapter 4)

To provide sufficient background and to understand the rationale behind the study design associated with each specific aim, the following sections of the introduction review relevant literature regarding the CYP superfamily of enzymes followed by subfamily organization and finally focusing on the CYP isoenzyme, CYP2A6, which is central to this dissertation work.

CYP450 Super Family

Cytochrome P450 (CYP) enzymes are a large group of enzymes responsible for a wide range of transformative activities in many biological species. In humans, there are 57 enzymes, 15 of which are currently known to metabolize drugs and xenobiotics. These 15 drug metabolizing enzymes perform 80% of the oxidative phase 1 reactions (Coleman, 2010). These enzymes (see Figure 1) are located in the lipophilic membrane of the smooth endoplasmic reticulum lying in wait for lipophilic drug compounds to flow right in. Important redox partners, P450 oxidoreductase (POR) and cytochrome b5, are present to provide the energy required for the oxidative reaction.

CYP enzymes are organized into categories, called families, based on amino acid sequence homology. In humans, 18 families exist for the 57 enzymes, yet there are 780 families across all biological species. A family has at least 40% of the full length amino acid sequence in common. Examples include CYP1, CYP2, CYP3 (Coleman, 2010).

Subfamilies have a minimum of 55% amino acid sequence homology and are denoted CYP2A, CYP2B, etc. Isoforms describe individual enzymes, yet carry 97% homology, and often differ in the binding site amino acid sequence, thus resulting in distinctive selectivity and function. Examples include CYP2A6 and CYP2A13 (Coleman, 2010).

An important and highly conserved characteristic of CYP is the heme containing active site. The heme contains iron and is able to gain or lose electrons to catalyze the oxidative or reduction reactions. Two electons, typically supplied by the redox partners described above, are required for a full cycle of the reaction. This active site is quite rigid, but surrounded by flexible complex binding areas, or "fingers," able to bind to substrates that fit within these fingers. These fingers contribute to the substrate diversity between isoforms.

CYP2 Family

The CYP2A family is the largest family of human CYP and metabolizes approximately half of drugs used therapeutically (Coleman, 2010). There are several subfamilies, including CYP2A, CYP2B, CYP2C, CYP2D, CYP2E.

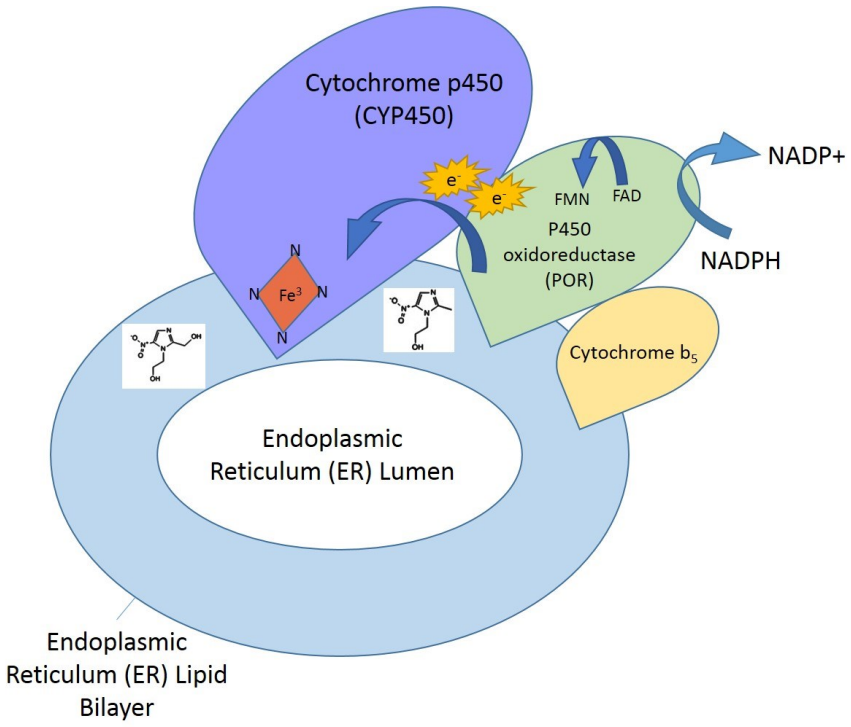

Figure 1. General structure of a CYP enzyme. A lipophilic substrate flows into the ER membrane and binds with the heme (Fe^3) active site of the CYP450 enzyme, where biotransformation occurs, generating a more hydrophilic metabolite.

CYP2A Subfamily

Three isoforms, CYP2A6, CYP2A7, CYP2A13, have been identified in the human CYP2A subfamily that originate on Chromosome 19. CYP2A7 is a pseudogene with no appreciable contribution to drug metabolism known at this time (Zanger & Schwab, 2013). CYP2A13 is expressed mainly in the nasal mucosa and respiratory tract and has been associated with the activation of tobacco-related procarcinogenic compounds, such as 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (nicotine-derived nitrosamine ketone (NNK) thus, playing a role in tumorigenesis associated with tobacco use (D'Agostino et al., 2008; Ting Su et al., 2000).

CYP2A6

General structure and substrate characteristics. CYP2A6 is a 494 amino acid enzyme that contributes up to 10% of the protein content of CYP in the liver (Di, Chow, Yang, & Zhou, 2009). It has also been identified in other human tissues such as small intestine, and lung (discussed in more detail in section the Expression and Distribution; Table 2). CYP2A6 metabolizes approximately 3% of drugs along with other environmental compounds. Substrates (see Table 1) include nicotine, cotinine, letrozole, and metronidazole. CYP2A6 contributes to a lesser degree in the metabolism of various compounds such as valproic acid and efavirenz. CYP2A6 is also involved in the bioactivation of human carcinogens such NNK and toxins such as aflatoxin B_1 and plays a role in bilirubin oxidation to biliverdin (Di et al., 2009; Zanger & Schwab, 2013). CYP2A6 is highly variable both in genetic structure and enzyme function i.e., activity. Greater than 40 variants in the *CYP2A6* gene have been characterized (McDonagh et al., 2012). Other sources of variability in expression or activity include induction or inhibition by pharmaceutics, endogenous compounds or dietary components, developmental contributions, and/or complex interactions with other hepatic enzymes (due to linkage disequilibrium, CYP450 co-factors). Variation has been associated with smoking behavior and cessation, lung cancer risk, and altered metabolism of several therapeutics (Tanner & Tyndale, 2017). These aspects will be discussed in more detail in subsequent sections.

CYP2A6 has the second smallest active site cavity volume of any human CYP with a known structure, thus typically accepting small molecules for oxidation. Only CYP2E1 has a smaller cavity volume, 190 $Å^3$ compared with CYP2A6's 260 $Å^3$ (Di et al., 2009). Crystallography and QSAR technology have provided insight to further define CYP2A6

substrate specificity and thus can be useful in predicting metabolic compatibility of future substrates. Noteworthy CYP2A6 features include an active binding site that accommodates a one- or two-ring chemical structure, typically planar in orientation. For substrates, the number of active site hydrogen bonds, preferably two, and π-π stacking interactions also appear to be of importance. A trend in the lipophilicity of substrates, as well as inhibitors (as described by log P) correlates well with binding affinity when combined in models with the aforementioned characteristics which echoes the logical assumption based on the known physio-chemical properties of most CYP enzymes (Lewis, Lake, Dickins, & Goldfarb, 2003). Using site-directed mutagenesis and binding titration assays, several amino acid residues important to the CYP2A6 binding site were identified to play a steric role in ligand affinity (DeVore, Smith, Wang, Lushington, & Scott, 2009). Taken together, these characteristics can be used to predict CYP2A6 binding capabilities of new chemical entities yet to be fully explored. It is important to note, however, that such characteristics should be used to guide predictions, but not limit the trial of potential chemical entities from CYP2A6 binding studies.

Several known chemical modulators of CYP2A6 have been described and are listed in Table 1. Mechanism-based inhibitors like selegiline shut down CYP2A6 activity most often in a time-, concentration- and NAPDH-dependent manner (Siu & Tyndale, 2008). Some mechanism-based inhibitors irreversibly debilitate the enzyme if present for a certain amount of time regardless of concentration, such as methoxsalen (Zhang, Kilicarslan, Tyndale, & Sellers, 2001). The competitive inhibitor tranylcypromine decreases CYP2A6 activity in a concentration dependent manner with Ki values ranging from 0.04-0.2 μM (Draper, Madan, & Parkinson, 1997; Stephens, Walsh, & Scott, 2012; Zhang et al., 2001).

Menthol, typically encountered in flavored cigarettes, has also been shown in some studies to inhibit CYP2A6 activity and will be discussed in greater detail in a later section. Typical CYP2A6 inducers along with their mechanism of induction are discussed in the following section and are listed in Table 1.

Table 1

Select CYP2A6 Substrates, Inducers, and Inhibitors

Substrates	Inducers	Inhibitors
nicotine (80% of 5-oxidation)	CITCO	tranylcypromine
cotinine (100% 3-hydroxylation)	rifampin	methoxsalen
metronidazole (≥ 96% 2-hydroxylation)	phenobarbital	ketoconazole
Efavirenz (7-hydroxylation)	estrogen	grapefruit juice
valproic acid	dexamethasone	menthol
letrozole (carbanol formation)	carbamazepine	isoniazid
Dexmedetomidine		pilocarpine
Tegafur (5-flurouracil formation)		selegiline
Bilirubin (oxidation to biliverdin)		diethyldithiocarbamate
Procarcinogens, such as NNK, NNAL NNN		
Toxins, such as aflatoxin B_1		

Note. Parentheses indicate percentage contribution from CYP2A6 for reaction listed

NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (nicotine-derived nitrosamine ketone); NNAL: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN, N-nitrosonornicotine; CITCO: 6-(4-chlorophenyl)imidazo(2,1-b)(1,3)thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
Select references: (Hukkanen, Jacob, & Benowitz, 2006; Maglich et al., 2003; Tanner & Tyndale, 2017; Zanger & Schwab, 2013)

CYP2A6 gene structure and regulation. As illustrated in Figure 2, CYP2A6 is located on the reverse strand of chromosome 19q13.2. It is a gene with 9 exons and 8 introns. It is known to be highly polymorphic with more single nucleotide polymorphisms (SNP) per kilobase (kb) than any other CYP, including CYP2D6 (Han et al., 2017).

CYP2A7 is a pseudogene in physical linkage with CYP2A6 and adds to the complexity of genomic interpretation (Fukami, Nakajima, Sakai, McLeod, & Yokoi, 2006).

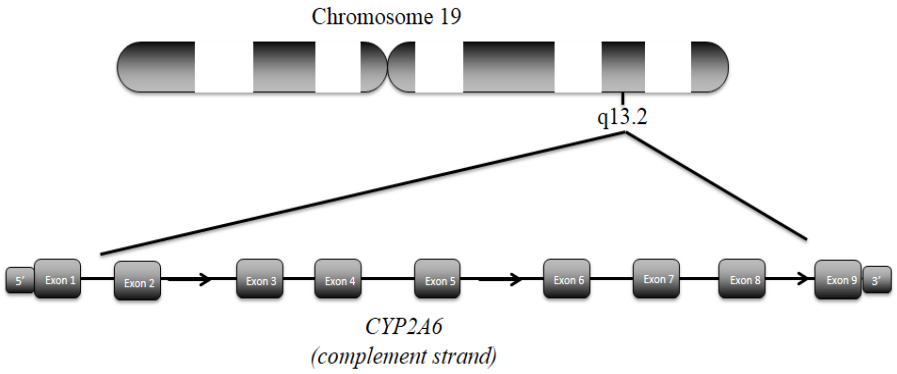

Figure 2. Gene structure of CYP2A6.

CYP2A6 is regulated by several nuclear receptors including pregnane X receptor (PXR), constitutive androstane receptor (CAR), estrogen receptor alpha (ERα) and glucocorticoid receptor (GR) (Di et al., 2009; Higashi et al., 2007; Itoh et al., 2006; Onica et al., 2008). Known ligands of PXR include rifampin and phenobarbital. CITCO is a potent CAR agonist, and phenobarbital can also activate this pathway of induction. Estrogen induces CYP2A6 expression via ERα. ERα is able to bind directly to the estrogen response element on the *CYP2A6* gene thus inducing expression (Higashi et al., 2007). This effect has been seen both *in vivo* and *in vitro*. Females have demonstrated increased nicotine clearance, primarily mediated by CYP2A6, compared with males. Additionally, adults on estrogen-containing contraceptives show even further increases in nicotine clearance compared with female non-users (Benowitz, Lessov-Schlaggar, Swan, & Jacob, 2006). This relationship was mirrored in an adult study measuring the caffeine metabolite ratio in urine as a marker of CYP2A6 activity (Sinues et al., 2008). Interestingly, another measure of CYP2A6 activity, the nicotine metabolite ratio (NMR), which measures trans-3-

hydroxycotinine/cotinine, did not differ between male and female adolescents aged 13-17 years or in those taking estrogen-containing contraception (Rubinstein, Shiffman, Rait, & Benowitz, 2013). Pregnancy, the period in a woman's life when the plasma levels of estrogen are at their highest, has also been associated with increased clearance of nicotine (Dempsey, Jacob, & Benowitz, 2002, Benowitz et al., 2006). *In vitro* work in hepatocytes showed that concentrations of estrogen seen in pregnancy (100 nM) led to significant induction of *CYP2A6* gene expression and activity (Choi, Koh, & Jeong, 2013). Levels of hepatic CYP2A6 mRNA and protein have demonstrated significantly greater abundance in female livers compared with male livers (Al Koudsi, Hoffmann, Assadzadeh, & Tyndale, 2010); however, there was no significant difference shown in enzyme kinetics tested in donor livers (Vmax and Km of nicotine). Of note, oscillating levels of estrogen associated with normal menstrual cycling do not appear to significantly impact CYP2A6 activity when evaluated by nicotine clearance (Hukkanen, Gourlay, Kenkare, & Benowitz, 2005). Taken together, the evidence suggests a concentration dependent effect of estrogen on CYP2A6 in adults to induce gene expression most likely through nuclear receptor (i.e., ERα) activation. Understanding the role of estrogen in the regulation of CYP2A6 in adolescents requires further investigation.

Dexamethasone activates growth hormone receptor (GR) with modulation by hepatic nuclear factor 4 (HNF4) alpha to induce CYP2A6 (Onica et al., 2008). One report showed an increase in CYP2A6 activity measured via the NMR in smokers taking the antiepileptic drugs oxcarbazepine and carbamazepine (Williams, Gandhi, & Benowitz, 2010). These medications are used for their mood stabilizing effects in the treatment of bipolar disorder and schizophrenia in addition to their use in seizure disorders, and are known to induce

CYP3A4. CYP3A4 and CYP2A6 share some regulatory pathways such as PXR and CAR; thus cross-over induction is certainly plausible.

CYP2A6 expression, distribution and variability. CYP2A6 is primarily expressed in the liver, but is also found at lower levels in the respiratory tract (e.g., nasal mucosa, trachea and lung) and various other human tissues (see Table 2). Large data repositories exist that catalog expression of myriad human genes and gene products. Methods such as microarray, RNA sequencing (RNAseq) and serial analysis of gene expression (SAGE) have been used by collaborative projects such as Genoytype-Tissue Expression Project (GTEx), Illumina, BioGPS and CGAP to describe mRNA expression in numerous human tissues and can have varying utility depending on the gene or protein of interest. When interpreting the relevance of such data reported in "bulk" for CYP enzymes, it is important to recall the homology between CYPs, particularly in subfamilies (e.g., CYP2A enzymes are 97% homologous). Understanding read length for the gene expression assays (i.e., RNAseq) would be important to assist in beginning to understand potential specificity of the findings to a particularly CYP isoenzyme, meaning longer read lengths of >100 nucleotides may protect against misalignment that can occur with short read lengths of 50-100. In recent years, proteomic methods utilizing mass spectrometry (MS) have attempted to determine protein expression in a variety of human tissues. Proteomic data sets share similar limitations with gene expression data sets. Specificity of the peptide fragments created by the MS/MS assay for a particular CYP is important to understand when interpreting the credibility of the expression reported. The methodology for both gene and protein expression detection should be discussed in detail in the primary literature reporting the results; yet, it may not be readily available in the large repositories aforementioned. Thus,

although freely available and easily accessible databases (such as genecards.org, proteinatlas.org, and pharmgkb.org) exist for the expression and distribution of CYP2A6, Table 2 references only the primary literature pertaining to each finding.

Correlation of *CYP2A6* gene expression, protein and activity. The relationship between gene expression, protein concentration, and activity for CYP2A6 is complex, with varying associations reported in the literature. In a recent study utilizing a large pool of human liver donors, CYP2A6 mRNA and protein were significantly correlated ($r=0.47$, $p<0.001$, $n=273$). In addition, CYP2A6 protein and activity were highly correlated ($r=0.88$, $p<0.001$, $n=329$) (Tanner et al., 2017). In pooled individual human liver microsomes ($n=67$-78), the tightest correlation was seen between CYP2A6 protein expression and activity ($r=0.77$, $p<0.001$), followed by mRNA and activity ($r=0.46$, $p<0.001$), and lastly, mRNA and protein ($r=0.40$, $p<0.001$) (Jamwal et al., 2017). This is similar to the correlation described in an *in vitro* report of 40 donor livers with CYP2A6 mRNA accounting for 45% of the variability in CYP2A6 protein ($r^2=0.45$, $p<0.001$). CYP2A6 protein and activity (measured by Vmax of nicotine c-oxidation) were also significantly correlated ($r^2=0.37$, $p<0.001$) (Al Koudsi et al., 2010). Notably, significant variability in relative CYP2A6 mRNA (1000-fold) and protein (100-fold) was observed. Another *in vitro* study evaluating 17 donor livers found poor correlation between CYP2A6 mRNA and protein with $r^2 <0.1$ (Ohtsuki et al., 2012). Although most of these relationships were statistically significant, quite a bit of variability in expression and/or activity of CYP2A6 was left to be explained. The most consistent associations are observed between CYP2A6 protein and activity. Discordance between mRNA and activity can be attributed to

Table 2

Expression and Distribution of CYP2A6 in Humans

	CYP2A6 mRNA	CYP2A6 protein	Sources
Liver	+ (fetal and adult)	+ 4% of adult HLM (coumarin 7-hydroxylase activity: ~25,000 pmol/min/mg HLM protein)	(Bièche et al., 2007; Jamwal et al., 2017)
Adrenal gland	+	+ (fetal only)	(Bièche et al., 2007; Wang et al., 2008)
Lung	+	±	(Bièche et al., 2007; Chiang, Wang, & Tsou, 2012; Crawford et al., 1998; Macé et al., 1998)
Nasal mucosa	+ (fetal and adult)	+ (fetal and adult)	(Ying Chen et al., 2003; Gu, Su, Chen, Zhang, & Ding, 2000; Koskela et al., 1999; Su, Sheng, Lipinskas, & Ding, 1996; Thornton-Manning et al., 1997)
Salivary gland	+		(Bièche et al., 2007)
Larynx	+		(Sarikaya, Bilgen, Kamataki, & Topcu, 2006)
Trachea	+	+	(Bièche et al., 2007; Chiang et al., 2012)
Thyroid	+		(Bièche et al., 2007)
Bone marrow	+		(Bièche et al., 2007)
Blood lymphocytes	+	+ (coumarin 7-hydroxylase activity: 0.25-1.75 pmoles/mg/min)	(Sharma et al., 2013; Siest et al., 2008)
Skin cells	+		(Janmohamed, Dolphin, Phillips, & Shephard, 2001; Saeki et al., 2002)
Skeletal muscle	+		(Bièche et al., 2007)
Esophagus	+		(Godoy et al., 2002)
Stomach	+		(Canturk et al., 2010)
Small intestine	+		(Bièche et al., 2007)
Large intestine	+ (carcinoma tissue)	+ (carcinoma tissue)	(Matsuda et al., 2007)
Spleen	+		(Bièche et al., 2007)
Bladder	+		(Bièche et al., 2007)
Kidney	+		(Bièche et al., 2007)
Breast	±	+ (coumarin 7-hydroxylase activity:1.5-4 pmol/mg/min)	(Hellmold et al., 1998; Iscan et al., 2001)
Ovary	+		(Bièche et al., 2007)
Endometrium	-	+	(Higashi et al., 2007; Hukkanen et al., 1998)
Placenta	+		(Bièche et al., 2007)
Testis	+		(Bièche et al., 2007)
Prostate	+		(Bièche et al., 2007; Zencir et al., 2008)

Note. +, detected; -, non-detected; ±, conflicting reports regarding detection vs. non-detection; blank fields lacked primary literature describing examination

a variety of regulatory factors including pre- and post-translational modifications. In addition, *in vitro* assays suffer from potential sample stability issues such as mRNA degradation that could affect correlation of mRNA to protein or activity. Activity may be affected by genetic polymorphisms that affect function rather than protein abundance (Zanger & Schwab, 2013). Thus, the specific evaluation of the CYP parameter of interest, most often activity when evaluating variability in drug metabolism, is important and any proxy should be viewed in light of appropriate caveats as mentioned above.

Polymorphic expression of CYP2A6. Human variability of CYP2A6 has been well documented *in vivo* and *in vitro* (Ting Su & Ding, 2004). Genetic polymorphisms contribute substantially to this variability. Recent investigations using next generation sequencing have provided insight into the expansiveness of single nucleotide polymorphisms in the *CYP2A6* gene. In addition, large genomic databases have contributed considerable knowledge to the distribution of genetic variants by race and ethnicity. Table 3 summarizes variants in the *CYP2A6* gene along with their functional consequences, if known, and is adapted from the study of nearly 57,000 unrelated individuals from five major populations (Zhou et al., 2017).

Table 3

Human CYP2A6 Variants

Allele	Associated variants	Exon/UTR	Nucleotide Δ	Amino acid Δ	Enzyme effect *in vitro*	Enzyme effect *in vivo*	Prevalence in humans
*1A	none					Normal	31-72%
*1B1	58bp gene conversion with CYP2A7	3'-UTR			increased mRNA stability	?; (linkage disequilibrium with decreased activity alleles)	11-70%
*1B2 (has also been called *1B)		3'-UTR	-1013A>G				
*1B3 (formally *1C)		3'-UTR	-395G>A				
*1X2A and B	Gene duplication	intron 8 and 5.2-5.6 kb 3'			increased mRNA expression		0-2%
*2	rs1801272	exon 3	51G>A; 1799T>A	L160H-missense		inactive-slow	<0.1-5%
*3	CYP2A6/CYP2A7 hybrid				?	?	
*4	Gene deletion					inactive	1-24%
*5	rs5031017	exon 9	6582G>T	G479V-missense	inactive	inactive	<0.1-1%
*6	rs4986891		1703G>A	R128Q-missense		decreased	<0.1%

Table continues

Allele	Associated variants	Exon/UTR	Nucleotide Δ	Amino acid Δ	Enzyme effect *in vitro*	Enzyme effect *in vivo*	Prevalence in humans
*7	rs5031016	exon 9	6558T>C	I471T-missense	decreased activity	decreased	0.1-13%
*8	rs28399946 8		6600G>T	R485L-missense		normal	0.30%
*9	rs28399433		TATA box(A>C)		decreased mRNA expression	decreased	8-23%
*10	rs5031016, rs28399468	exon 9	6558T>C; 6600G>T	I471T, R485L-missense		decreased	0.1-4%
*11			3391T>C	S224P	decreased	decreased	
*12		CYP2A6 (exons 3-9)-CYP2A7 (exons 1-2) hybrid		10 aa substitutions		decreased	0-1%
*13			-48T>G; 13G>A	G5R			
*14	rs28399435		51G>A; 86G>A	S29N-missense			0.1-4%
*15			-48T>G; 2134A>G	K194E			
*16	rs56256500		2161C>A	R203S			<0.1%
*17	rs28399454	exon 7	209C>T; 1779G>A; 4489C>T; 5065G>A; 5163G>A; 5717C>T; 5825A>G	V365M-missense	decreased	decreased nicotine c-oxidation); normal coumarin 7-hydroxylation	0.6-11%

Table continues

Allele	Associated variants	Exon/UTR	Nucleotide Δ	Amino acid Δ	Enzyme effect *in vitro*	Enzyme effect *in vivo*	Prevalence in humans
*18A/B	rs1809810	exon 8	5668A>T	Y392F	decreased		0.6-2%
*19	rs5031016, rs1809810		5668A>T; 6354T>C; 6558T>C	I471T, Y392F-missense	decreased		0.1-1%
*20		2 nucleotide deletion in exon 4	51G>A; 2141_2142delAA; 2296C>T; 5684T>C; 6692C>G	Frameshift (196)	inactive	decreased	0-2%
*21	rs6413474	exon 9	51G>A; 6573A>G	K476R		decreased	0.1-3%
*22	rs60605885, rs60563539		51G>A; 1749C>G; 1798C>A	D158E, L160I-missense			0.10%
*23	rs56256500	exon 4	2161C>T	R203C-missense	decreased	decreased	0.1-2%
*24	rs143731390, rs72549435	exon 2, 9	-1301A>C; -1289G>A; -1013A>G; 51G; 578A>G; 594G>C; 720G>A; 1137C>G; 1381_1382CT>TC; 1481_1486delCTCTCT; 1620T>C; 2483G>A; 3225A>G; 5668A; 6218A>G; 6282A>G; 6293T>C; 6354T>C; 6458A>T; gene conversion in 3'-UTR; 6782C>G; 7160A>G	N438Y, V110L-missense		decreased	0.1-2%

Table continues

Allele	Associated variants	Exon/UTR	Nucleotide Δ	Amino acid Δ	Enzyme effect *in vitro*	Enzyme effect *in vivo*	Prevalence in humans
*25	rs28399440	exon 3	-1301A>C; -1289G>A; -745A>G; 22C>T; 51G; 768A>T; 1620T>C;1672T>C; 2296C>T; 2483G>A; 2605G>A; 2921G>A; 2994T>C; 4636A>C; 5668A; 6586T>C; 6692C>G; 7160A>G	F118L-missense		decreased	1%
*26	rs59552350		-1301A>C; -1289G>A; -745A>G; 22C>T; 51G; 1165G>A; 1620T>C; 1672T>C; 1703G>T; 1710C>T; 1711T>G; 2296C>T; 2483G>A; 2994T>C; 4071delA; 4636A>C; 5668A; 6115C>T; 6586T>C; 6692C>G; 7160A>G	S131A-missense; F118L; R128L	decreased	decreased	<0.1-0.4%
*27			-1301A>C; -1289G>A; -745A>G; 22C>T; 51G;1620T>C; 1672T>C; 2162_2163GC>A; 2296C>T; 2483G>A; 2994T>C; 3872G>A; 4071delA; 4636A>C; 5668A; 5857T>A; 6586T>C; 6692C>G; 7160A>G	F118L; R203Frameshif	decreased		

Table continues

Allele	Associated variants	Exon/UTR	Nucleotide Δ	Amino acid Δ	Enzyme effect *in vitro*	Enzyme effect *in vivo*	Prevalence in humans
*28	rs28399463, rs8192730	exon 8	-1269T>C; 51G>A; 656G>T; 1620T>C; 4681T>G; 5668A; 5738C>T; 5745A>G;5750G>C; 6354T>C; 6361C>A; 6385G>T; 6389C>G; 6390T>C; gene conversion in 3'-UTR; 6782C>G; 7160A>G	N418D, E419D-missense		decreased	<0.1-2%
*35	rs143731390	exon 9	-1301A>C; -1289G>A; -1013A>G; 720G>A; 1137C>G; 1620T>C; 2483G>A; 3225A>G; 6218A>G; 6282A>G; 6293T>C; 6354T>C; 6458A>T; gene conversion in the 3' flanking region; 6782C>G; 7160A>G	N438Y-missense	decreased	decreased	4-15%
*36			-1301A>C; -1289G>A; -745A>G; 22C>T; 1620T>C; 4084delA;6458A>T; 6558T>C; gene conversion in the 3' flanking region; 6782C>G; 6835C>A; 6999T>C; 7160A>G	N438Y; I471T			

Table continues

Allele	Associated Variants	Exon/UTR	Nucleotide Δ	Amino acid Δ	Enzyme effect *in vitro*	Enzyme effect *in vivo*	Prevalence in humans
*37			-1301A>C; -1289G>A; -745A>G; 22C>T; 1620T>C; 4084delA; 6354T>C; 6458A>T; 6558T >C;6600G>T; gene conversion in the 3' flanking region; 6782C>G; 6835C>A; 6936_6937insCACTT; 6961_6962insGAAAAG; 6989A>G; 6999T>C; 7160A>G	N438Y; I471T; R485L			
*38			5023A>G	Y351H			
*39	rs143690364		171C>A; 468G>A; 1779G>A; 5717C>T	V68M		decreased	<0.1%
*40			144G>A; 1767C>G; 3492C>T; 5738C>T	I149M		decreased	
*41	rs140471703		51G>A; 507C>T; 3515G>A	R265Q	inactive	inactive	<0.1-0.2%
*42			51G>A; 3524T>C; 5684T>C	I268T		decreased	
*43			4406C>T	T303I		decreased expression; unchanged activity	
*44			51G>A; 5661G>A; 5738C>T;5745A>G; 5750G >C	E390K: N418D; E419D		decreased expression; increased activity	
*45			51G>A; 4464G>A; 6531T>C	L462P		decreased expression; decreased activity	

Adapted from Tanner & Tyndale, 2017; Zhou et al., 2017; www.pharmvar.org (formerly cypalleles.ki.se)

Certain allelic variants such as *17 are associated with substrate specific differences in activity. The rate of coumarin 7-hydroxylation as well as reaction velocity (V_{max}) observed in CYP2A6*1/*17 *in vitro* enzyme constructs do not differ from the wild type enzyme. However, the rate of *in vitro* nicotine c-oxidation as well as cotinine/nicotine and 3-hydroxycotinine/cotinine ratios in CYP2A6*1/*17 patients are reduced by ~50% compared to wild type individuals (Fukami et al., 2004; Ho, Mwenifumbo, Zhao, Gillam, & Tyndale, 2008).

Several studies have established an association between CYP2A6 polymorphisms and health risks. CYP2A6 allelic variants conferring decreased activity are associated with variable smoking behavior, self-reported nicotine dependence, and quit ability (Chenoweth & Tyndale, 2017; Tanner & Tyndale, 2017). Smoking behavior and addiction are multifactorial, and other biological sources of variability, including nicotinic acetylcholine receptors, are an important piece of the puzzle. Existing literature is inconsistent about the directional impact of allelic variants on certain aspects of smoking behavior. This may be due to the difference of tobacco-related measures assessed in different studies and poses a challenge in generalizing findings or comparing results between studies. Examples of various measures of tobacco use and dependence include cigarette consumption per day (CPD), nicotine dependence scales (e.g., Fagerström Test for Nicotine Dependence), and smoking topography (e.g., puff volume and frequency). A recent meta-analysis pointed to the association between CYP2A6 polymorphism and daily cigarette consumption, yet no difference in measures of tobacco dependence was demonstrated (Pan, Yang, Li, & Jia, 2015).

Emerging evidence suggests that *CYP2A6* genotype may be beneficial in the diagnostic consideration of tobacco cessation therapy. For example, extensive metabolizers (e.g., those with normal enzyme activity) may find more success if given a pharmacologically-augmented quit strategy using varenicline or bupropion. In contrast, poor metabolizers (e.g., those with decreased enzyme activity) may do well with nicotine replacement therapy such as the patch or gum (Chenoweth & Tyndale, 2017; Saccone et al., 2018; Schuit et al., 2017).

Groups have also looked at risk of cancer in individuals with CYP2A6 allelic variants or phenotypic variation. Those with genetic variants conferring decreased activity are associated with lower risk of lung cancer compared with normal activity, potentially due to the lower exposure to carcinogenic compounds activated by CYP2A6 (Patel et al., 2016; Wassenaar et al., 2015). The association is strengthened with the comparison of extensive or "normal" metabolizers (*1/*1) to individuals with full *CYP2A6* gene deletion (*4/*4) (Z.-B. Liu, Shu, Wang, Jin, & Lou, 2013; Park et al., 2017). The association between *CYP2A6* genotype and lung cancer risk persisted after correction for cigarette consumption (which is often linked with activity) in most ethnic groups, although differential effects have been described (Bergen et al., 2015; Park et al., 2016, 2017). One report identified an association with a particular CYP2A6 SNP resulting in a missense mutation that was associated with pancreatic cancer (Hocevar et al., 2014).

Exposure and response to chemotherapeutics have also been associated with *CYP2A6* genotype and activity. Exposure to the antiretroviral efavirenz appears to be associated, at least in part, with CYP2A6. An association with treatment outcomes such as viral suppression has yet to be described (McDonagh, Lau, Alvarellos, Altman, & Klein,

2015). Exposure to letrozole, used to treat breast cancer, has been significantly associated with *CYP2A6* genotype. *CYP2A6* variant alleles conferring reduced function have been associated with decreased metabolism of letrozole both in patients with breast cancer and in healthy post-menopausal women (Desta et al., 2011; Tanii, Shitara, & Horie, 2011). The impact of CYP2A6 variation on breast cancer treatment outcomes is yet to be determined. Tegafur is often administered in a multi-drug regimen to treat gastric cancer and undergoes activation of 5-fluorouracil by CYP2A6. Studies have investigated the association of efficacy of tegafur containing regimens with *CYP2A6* genotype and found increased CYP2A6 activity is associated with better response and longer survival (Kong et al., 2009). CYP2A6 is responsible for the activation of the prodrug artesunate and contributes to the clearance of artemisinin, thus the consequences of variation differ based on the distinct roles that CYP2A6 plays in the metabolism of these antimalarial medications (e.g., decreased exposure vs. increased toxicity). Studies with thorough allelic inquiry are needed to determine if reduced function genotypes yield decreased therapeutic efficacy of artesunate (Tanner & Tyndale, 2017).

Much is left to be elucidated regarding the importance of CYP2A6 genetic polymorphism and variability on health risks and treatment outcomes in sub-populations. Further studies will add to the body of evidence determining how actionable this genetic information may be at the individual level.

CYP2A6 ontogeny. Current evidence suggests that age is only minimally impactful on CYP2A6 variability. Tanner et al. (2017) described a weak, but significant trend (r=0.2; p<0.001) towards increase in activity *in vitro* with increasing age when activity was measured via cotinine formation from nicotine. In primary human hepatocytes, donors aged

>20 years had 1.5x the CYP2A6 activity of those aged <20 years (Parkinson, Mudra, Johnson, Dwyer, & Carroll, 2004). Another study in liver samples from 44 donors ranging in age from 2-64 years showed no relationship between age and CYP2A6 protein or activity (measured by Vmax of nicotine c-oxidation) (Al Koudsi et al., 2010). In an *in vitro* study comparing liver samples from neonates and infants aged <1 year to patients aged >1 year, CYP2A6 protein was detected in all samples with no difference in concentration of CYP2A6 protein (Tateishi et al., 1997). This study used a relatively small number of samples per group (n=10), and activity was not reported.

Clinical studies have also tried to look at the relationship of age to CYP2A6 activity. One study in children evaluated the effect of age on CYP2A6 clearance via cotinine half-life and found that age between 2.8 months and 6.8 years did not have an effect, yet *CYP2A6* genotype did (D. A. Dempsey et al., 2013). Dempsey first studied the elimination half-lives of both nicotine and cotinine in newborns on their first day of life and found that elimination of nicotine, but not cotinine differed significantly compared with adults (D. Dempsey, Jacob, & Benowitz, 2000). As mentioned previously, CYP2A6 is responsible for approximately 80% of nicotine biotransformation and 100% of cotinine metabolism, thus suggesting that the similarity in cotinine half-lives between newborns and adults speaks more specifically to CYP2A6 ontogeny.

A study in adults aged 18-57 years utilizing caffeine to probe CYP2A6 activity found that increasing age was predictive of increased CYP2A6 activity ($p<0.001$) (Sinues et al., 2008). Metronidazole clearance when measured by half-life appears to be prolonged by 2-fold in neonates compared with adults (Upadhyaya, Bhatnagar, & Basu, 1988). Another study evaluating the clearance of metronidazole found that half-life was significantly

associated with post-menstrual age (PMA) in pre-term infants in an intensive care setting. In this study, the most immature infants (PMA <32 weeks) demonstrated twice the half-life of those with a PMA >32 weeks. Yet, even in the latter cohort of infants (PMA >32 weeks), the reported half-life was still nearly twice that of adults (Suyagh et al., 2011). Half of the infants in this study were treated therapeutically with caffeine, which has been shown to inhibit CYP2A6 activity both *in vitro* and in smokers (Woodward, 2008). The relevance of this interaction in infants treated concurrently with metronidazole and caffeine is yet to be fully investigated, and adds to the complexity of understanding the ontogeny of CYP2A6 in a clinical setting.

In summary, CYP2A6 protein appears to be present at term-birth and at least partially functional. The characterization of any latency in CYP2A6 activity in the very young (i.e., birth to two months) is yet to be fully determined.

Other sources of CYP2A6 variability. Variability in activity remains to be accounted for even after assessment of genotype, age, and other factors. Therefore, investigation has moved into exogenous factors such as the foods we eat (i.e., dietary substances) and endogenous factors other than those mentioned above that might affect activity.

Dietary substances. Scientists have speculated that dietary substances that may be encountered intermittently via food or through herbal supplements may affect CYP2A6, and there is some evidence to suggest certain compounds may interact with CYP2A6 and lead to alterations in activity. Caffeine, a substrate of CYP2A6, also has the ability to inhibit this enzyme (Woodward, 2008). Grapefruit juice, known to inhibit CYP3A4 enzymes, has demonstrated a decrease in the rate of nicotine c-oxidation, mediated by CYP2A6, in

humans (Hukkanen et al., 2006). Yet, grapefruit juice had no effect when caffeine was administered as the probe drug for CYP2A6 activity (de Waard et al., 2008). Menthol, encountered in flavored cigarettes, has been associated with decreased CYP2A6 activity *in vivo* measured by 3-hydroxycotinine/cotinine ratio (NMR) in plasma compared against non-mentholated cigarettes; however, *in vitro* studies are less clear about the ability of menthol to interact with CYP2A6 (Fagan et al., 2016; Kramlinger, von Weymarn, & Murphy, 2012; MacDougall, Fandrick, Zhang, Serafin, & Cashman, 2003). Isoflavones like those found in soy products showed a significant reduction in the cotinine/nicotine ratio in a small study of Japanese volunteers (Nakajima et al., 2006). In a study of healthy females looking specifically at the effect of genistein, an isoflavone thought to have estrogenic products, CYP2A6 activity probed with caffeine was significantly increased (Yao Chen et al., 2011). More studies are needed to determine if isoflavones found in soy products affect CYP2A6 activity in a meaningful way if encountered during routine dietary consumption. Likewise, cinnamaldehyde, a component of cinnamon powder taken by some patients as a supplement to affect diabetes, has exhibited CYP2A6 inhibitory effects *in vitro* (Chan et al., 2016). The concentrations used in the study may perhaps be encountered after high dosing of cinnamon powder supplementation; however, studies in humans would be needed to determine the extent of such an effect on therapeutic substrates of CYP2A6.

Potential co-regulators. Enzyme co-regulators have been investigated as a potential source of variability in function. Aldo-keto reductase 1D1 (AKR1D1), a bile acid synthase, may indirectly modulate CYP2A6 through nuclear receptor regulation of CAR and PXR. *In vitro* evidence from liver bank samples described a significant correlation between AKR1D1

mRNA and CYP2A6 mRNA (r=0.57, p<0.001), protein (r=0.30, p<0.001), and activity (r=0.34, p<0.001) (Tanner et al., 2017).

POR protein is known co-regulator of CYP450 both in physical proximity and function (i.e., donating electrons to power enzyme reactions). POR as a contributor to CYP2A6 variability has been investigated *in vitro*. In samples from a human liver bank, POR protein content was correlated with CYP2A6 activity (r=0.45, p<0.001) (Tanner et al., 2017). Genetic polymorphism of POR has also been associated with CYP2A6 activity. Investigations found a single nucleotide polymorphism (SNP) in *POR* was associated with the NMR *in vivo* in normal CYP2A6 metabolizers by genotype; however, it was not correlated with NMR overall, thus suggesting minimal contribution of POR genetic variation to CYP2A6 variability (Chenoweth et al., 2014).

Another potential factor contributing to CYP2A6 variability is regulation by microRNA (miRNA). A miRNA is a small non-coding RNA molecule, approximately 22 nucleotides in length, that acts on RNA to silence transcription or alter translation to protein. Expression of miRNA is a dynamic process affected by stress, disease, and environmental influences. In primary human hepatocytes, miR-126* overexpression decreased CYP2A6 protein levels by 26%. Inhibition of miR-126* significantly increased CYP2A6 protein levels 2-fold over control (Nakano et al., 2015). This suggests miR-126* may play a role in CYP2A6 inter-individual variability. Further examination, particularly *in vivo*, would be important to understand the impact on variability in humans.

CYP2A6 Probe Drugs

Existing CYP2A6 probe drugs. Several phenotyping probes exist to study variability in CYP2A6 activity. As previously described, variability may be influenced by

genetic polymorphisms, environmental exposures, concurrent medications, or endogenous compounds. Measuring activity with a phenotyping probe provides a summary of the many factors at play rather than a snapshot of one potential factor, such as genotype. Important considerations when selecting a probe drug include specificity (for the reaction measured), safety, tolerability, cost, and availability of the compound.

Current *in vivo* probes for CYP2A6 include nicotine, coumarin, and caffeine. Each of these existing probes carries significant limitations, either due to safety concerns, feasibility for use in children, or enzyme specificity. The current "gold standard" for determining CYP2A6 phenotype is the use of nicotine to determine the ratio of either cotinine/nicotine (ct/nic) or 3-hydroxycotinine/cotinine (3HC/COT) in plasma or urine (D. Dempsey et al., 2004; Peamkrasatam et al., 2006). This has been accomplished in non-smokers through administration of oral labeled nicotine and/or nicotine gum. In current smokers, 3HC/COT can be measured any time once steady state is achieved, offering a convenient, time-independent measure. Importantly, nicotine is not legal for use in children under the age of 18 in the majority of the world, in large part due to its highly addictive and toxic characteristics. Some groups have administered deuterium-labelled cotinine directly to children (oral) or adults (intravenous) for determination of 3HC/COT; yet, this is not a compound readily available or easy to access (Benowitz, St Helen, Dempsey, Jacob, & Tyndale, 2016; D. A. Dempsey et al., 2013; Zhu et al., 2013).

Coumarin is a toxic anticancer agent no longer approved for use in the United States due to safety concerns. Coumarin is also rapidly metabolized, therefore, accurate capture of necessary concentrations in biologic fluid proves challenging.

Caffeine via the 8-hydroxylation of paraxanthine (17U/17X) has also been proposed as a probe for CYP2A6 activity; however, caffeine is also a substrate of CYP1A2, N-acetyltransferase 2 (NAT2) and xanthine oxidase (XO), yielding poor specificity (Hakooz, 2009).

Metronidazole as a novel CYP2A6 probe. Development of a novel, safe, well-tolerated probe with a reaction high specificity for CYP2A6 would serve to further the understanding of variability in therapeutic response to drugs metabolized through this pathway and widen the path towards precision medicine. Metronidazole is a compound that may serve as good probe drug for CYP2A6 by overcoming some of the challenges of the existing probe drugs. Recent evidence has demonstrated that the biotransformation of metronidazole to 2-hydroxymetronidazole is highly specific for CYP2A6. 2-hydroxymetronidazole is the most abundant metabolite accounting for approximately 60% of the fraction metabolized (see Figure 3). Other metabolites described include metronidazole glucuronide (accounting for <15%) and metronidazole acetic acid (accounting for 5-25%). At concentrations consistent with those seen in human plasma, ≥96% of 2-hydroxymetronidazole formation is catalyzed by CYP2A6 (Pearce, Cohen-Wolkoweiz, Sampson, & Kearns, 2013). Furthermore, CYP2A6 inhibitors, both competitive and mechanism-based, nearly abolish 2-hydroxymetronidazole formation in human liver microsomes. Additionally, targeted anti-CYP2A6 antibodies prevent any 2-hydroxymetronidazole formation (Pearce et al., 2013). Other CYP enzymes did not contribute to 2-hydroxymetronidazole formation within the range of therapeutic concentrations, nor was formation of this metabolite affected by inhibition or antibody blockade of other CYPs.

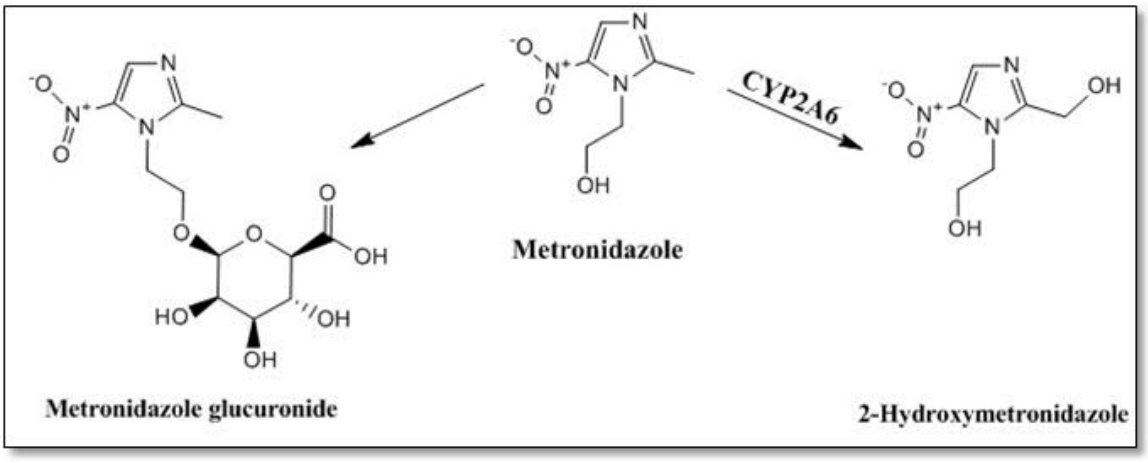

Figure 3. Structural schematic of the biotransformation of metronidazole. Metronidazole undergoes 2-hydroxylation by CYP2A6 resulting in the active metabolite, 2-hydroxymetronidazole. Glucuronide formation from the parent compound represents a minor pathway (approximately 15%) in the metabolism of metronidazole. Another minor metabolite, metronidazole, acetic acid, has been reported in some, but not all patients exposed to metronidazole *in vivo*.

Metronidazole is an antimicrobial with a unique spectrum of activity against both parasites and anaerobic bacteria (Samuelson, 1999). Metronidazole is the drug of choice for treatment of *T. Vaginalis*, the most common sexually transmitted disease globally, and is widely available for clinical use (Bouchemal, Bories, & Loiseau, 2017). Metronidazole is also a vital component of the treatment of *C. difficile*, amebic infections, and *H. pylori* and is used across the lifespan from neonates to elderly (Samuelson, 1999). Metronidazole has been used therapeutically in the United States and abroad for over 50 years with significant evidence of favorable safety and tolerability profile, particularly at low oral doses.

Validation of metronidazole as a novel CYP2A6 phenotyping probe drug would allow for mitigation of potential risks associated with the currently available probe drugs, nicotine and coumarin, and lack of specificity associated with caffeine. In addition, it is imperative to further investigate the substrate specific response of certain CYP2A6 allelic

variants in humans, particularly regarding metronidazole, to add to the growing body of knowledge that supports continued expansion and implementation of precision medicine.

Summary and Introduction to Dissertation

CYP2A6 is a polymorphically expressed enzyme responsible for the biotransformation of nicotine, metronidazole, efavirenz, coumarin, and the activation of procarcinogenic compounds. Genetic variability of *CYP2A6* is quite prevalent, ranging from 28-69% depending on race, with East Asians as the most frequent carriers of allelic variants (Zhou et al., 2017). Decreased activity allelic variants are most predominant with phenotypic impact ranging from intermediate (~75% activity) to slow (<50% of activity) compared with wild type (Pan et al., 2015). Studies have linked decreased CYP2A6 activity with differences in smoking behavior and lung cancer risk (Chenoweth et al., 2016; Derby et al., 2008; Fagan et al., 2015; Y. Liu, Xu, Li, Chen, & Guo, 2013; Moolchan, Franken, & Jaszyna-Gasior, 2006; Wassenaar et al., 2015). Phenotyping probes for CYP2A6 have been used to understand variability in activity as well as the genotype-phenotype relationship. Current *in vivo* probes for CYP2A6 include nicotine, coumarin, and caffeine. Each of these existing probes carries significant limitations, either due to safety concerns, feasibility for use in children, or enzyme specificity. Development of a novel, safe, well-tolerated probe with a reaction that is high specificity for CYP2A6 would enhance the armamentarium available for understanding variability in therapeutic response to drugs metabolized through this pathway, thus pushing towards precision medicine. The following research will evaluate the administration of metronidazole followed by measurement of 2-hydroxymetronidazole/metronidazole to serve as a biomarker for CYP2A6 activity in humans compared with the gold standard, nicotine metabolite ratio. To provide a clear

pathway for justification of metronidazole metabolite ratio as a specific biomarker for CYP2A6, *in vitro* experiments were undertaken to understand any relevant inhibitor or inducer influence of metronidazole and nicotine. The information gained from the enclosed studies will provide evidence regarding the use of metronidazole as a safe, novel CYP2A6 phenotyping probe drug in humans. The data generated will be relevant and immediately applicable to the development of a future, complementary study in children using metronidazole.

CHAPTER 2

EFFECTS OF METRONIDAZOLE AND NICOTINE ON CYP2A6 ACTIVITY AND mRNA EXPRESSION *IN VITRO*

Introduction

CYP2A6 is a polymorphic enzyme responsible for the biotransformation of approximately 3% of medications along with clearance of nicotine and activation of procarcinogens found in tobacco. Variations in CYP2A6 have been linked to lung cancer susceptibility and smoking behavior (Tanner & Tyndale, 2017) along with differential exposure to the antiretroviral medication, efavirenz (McDonagh et al., 2015). Inter-individual variability up to 275-fold has been reported for CYP2A6 and has been shown to be influenced by genetic polymorphisms and exposure to both endogenous and exogenous compounds, thus underscoring the importance of a tool, such as a phenotyping probe, that is able to provide insight into this variability in humans to potentially guide risk assessment and therapeutic decisions (Di et al., 2009; Tanner et al., 2017).

The current gold standard CYP2A6 phenotyping probe, nicotine, is catalyzed by CYP2A6 to cotinine and, further, to trans-3-hydroxycotinine. Measurement of metabolite/parent ratio is convenient, particularly in current smokers who are frequently exposed to nicotine. Yet, having another option to understand variability of CYP2A6 is attractive to avoid unnecessary exposure to nicotine and/or cotinine in those who would not otherwise be exposed. Metronidazole is selectively converted to 2-hydroxymetronidazole by CYP2A6 and has been proposed as a specific phenotyping probe for this enzyme in humans (Pearce et al., 2013). Metronidazole is an antimicrobial with a unique spectrum of activity

against both parasites and anaerobic bacteria and has been used in the treatment of many diseases in both adults and pediatric patients worldwide (Samuelson, 1999).

Criteria has been proposed to guide the selection and validation of medications that may be used as phenotyping probes in humans. The candidate probe should reflect induction and inhibition of the enzyme of interest and "not be relevantly influenced by other factors," but rather a real-time reflection of complex factors involving biotransformation of the pathway (Faber et al., 2005; Streetman et al.,2000). Since both metronidazole and nicotine are substrates of CYP2A6, evaluation of their ability to inhibit or induce CYP2A6 *in vitro* is important to determine. To our knowledge, there have been no reports that have fully characterized the potential for metronidazole to induce or inhibit CYP2A6. Prior reports have evaluated the ability of nicotine to inhibit CYP2A6, but limited data is available regarding its ability to induce CYP2A6 *in vitro.* Therefore, the objective of this study was to examine the effects of metronidazole and nicotine on CYP2A6 activity and mRNA expression *in vitro.* Data may be used to inform an *in vivo* study designed to determine the validity of metronidazole as a CYP2A6 phenotyping probe compared with the gold standard, nicotine.

Methods

Chemicals and Reagents

CITCO, i.e., 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime, metronidazole, and nicotine, were purchased from Sigma-Aldrich (St. Louis, MO) whereas 2-hydroxymetronidazole, D_4-2-hydroxymetronidazole and D_3-cotinine were purchased from Toronto Research Chemicals (Toronto, ON). Cryopreserved pooled primary human hepatocytes (PHHs; n =10, mixed gender), culture media, kits for

isolating and thawing hepatocytes, and pooled human liver microsomes (HLMs; n = 16 donors) were obtained from Xenotech LLC (Lenexa, KS). PHHs isolated from the livers of four, non-smoking female donors (aged 20-33 years) were purchased from Triangle Research Labs (Raleigh, NC). All other chemicals were of reagent grade or higher.

Inhibition of Nicotine C-oxidation

Pooled HLMs (10 µg protein) and cytosol (50 µg protein) were incubated in 100-µL reactions containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM), and nicotine (12.5-200 µM) in the presence or absence of metronidazole (0-300 µM). Cytosol was added to the incubations because the conversion of nicotine to cotinine is a two-step reaction, with CYP2A6 responsible for the conversion of nicotine to an intermediate, nicotine-Δ 1'-(5')-iminium ion, followed by conversion to cotinine by aldehyde oxidase. Substrate concentrations of nicotine were chosen to bracket an empirically determined K_m (56.7 µM) in pooled HLMs (approximately ¼ K_m, ½ K_m, K_m, $2K_m$, $4K_m$). Reactions were initiated by the addition of an NADPH-generating system consisting of NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 IU/ml) at 37°C in a shaking water bath and terminated after 30 minutes by the addition of ice-cold methanol (100 µl) containing the internal standard, cotinine-d_3. Samples were centrifuged at 10,000 g x 10 minutes to precipitate protein, and an aliquot (5 µl) of the supernatant was analyzed by UPLC-MS/MS using a method adapted from Dobrinas (Dobrinas et al., 2011) to determine concentrations of cotinine. Experiments were performed in triplicate with duplicate technical replicates. Reactions were conducted under conditions where product formation was directly proportional to protein concentration and time.

Inhibition of Metronidazole 2-Hydroxylation

Pooled HLMs (25 μg protein) were incubated in 100-μL reactions containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM), and metronidazole (75-1200 μM) in the presence or absence of nicotine (0-50 μM). Substrate concentrations of metronidazole were chosen to bracket an empirically determined K_m (300 μM) in pooled HLMs (approximately ¼ K_m, ½ K_m, K_m, $2K_m$, $4K_m$). Positive control experiments were also completed using the potent CYP2A6 competitive inhibitor, tranylcypromine (0.05-4 μM). Reactions were initiated by the addition of an NADPH-generating system consisting of NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 IU/ml) at 37°C in a shaking water bath and terminated after 60 minutes by the addition of ice-cold methanol (100 μl) containing the internal standard, 2-hydroxymetronidazole-d_4. Samples were centrifuged at 10,000 g x 10 minutes to precipitate protein and an aliquot (3 μl) of the supernatant was analyzed by UPLC-MS/MS to determine concentrations of 2-hydroxymetronidazole (Stancil, van Haandel, Abdel-Rahman, Pearce, 2018). Experiments were performed in triplicate with duplicate technical replicates. Reactions were conducted under conditions where product formation was directly proportional to protein concentration and time.

Culture of Primary Human Hepatocytes

PHHs were thawed according to the manufacturer's protocol, counted and plated on collagen I coated 6-well plates (BD BioCoat; Bedford, MA) at a seeding density of 1 million cells/ml (pooled PHHs) and 1 million cells/well (individual donors). After cells attached to the plates (2-6 hours), Corning Matrigel (Tewksbury, MA) was overlayed at 0.25 mg/ml. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. After an

overnight incubation, pooled PPHs (used to optimize experimental conditions) were treated with vehicle control (0.1% DMSO), positive control (Constitutive Androstane Receptor agonist, CITCO, 500 nM), or nicotine (0.3 μM corresponding to the maximum concentration seen in plasma of human smokers and 1 μM). After an overnight incubation, individual donor PHHs were treated with vehicle control (0.1% DMSO), positive control (Constitutive Androstane Receptor agonist, CITCO, 500 nM), or metronidazole [300 μM] for 72 hours. Media containing test compounds was replaced every 24 hours. Following the 72-hour treatment period, PHHs were triple washed with phosphate-buffered solution, and fresh media containing metronidazole [100 μM] was added for activity *in situ* assay. Aliquots (200 μl) of media were removed at 0.5, 2, 4, 6, 8 hours. Sufficient media remained after removing each aliquot to cover cells. After removal of media, aliquots were centrifuged. Supernatant was then removed and evaporated to dryness. Samples were reconstituted in 100 μl 10% ACN and concentrations of metronidazole and 2-hydroxymetronidazole were determined by a validated UPLC-MS/MS method (Stancil et al., n.d.). Experiments were performed in triplicate.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from PHHs after the 8-hour activity *in situ* assay time point using Qiagen AllPrep DNA/RNA mini kits (Qiagen, Valencia, CA). cDNA was synthesized using a High Capacity cDNA kit (Life Technologies; Carlsbad, CA). Quantitative real-time polymerase chain reaction amplification of cDNA (corresponding to 30 ng of total RNA) was performed in an 8-μl reaction mixture containing 1X PerfeCta QPCR Supermix (Quantabio, Beverly, MA) using the TaqMan Gene Expression Assay for CYP2A6 (HS00868409_s1) and GAPDH. Cycling conditions were initiated with denaturation at 95°C

x 3 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were generated by serial dilutions of plasmids containing PCR amplicons from 10 to 10^7 copies from which transcript numbers were calculated by linear regression. Reactions were performed in triplicate and results are expressed as molecules *CYP2A6*/ng total RNA after correction with molecules of *GAPDH*.

Data Analysis

Kinetic constants (Km and Vmax) were determined from lines of best fit on Eadie-Hofstee plots and verified by visual inspection. Determination of Ki values were calculated for each concentration of inhibitor using the formula:

$$K_i = \frac{K_m \times [I]}{(K_{mapp} - K_m)}$$

where [I]=inhibitor concentration and $K_{mapp} = K_m(1 + [I]/K_i)$ and is the apparent K_m observed in the presence of inhibitor. The mean K_i value was then reported as mean ± SD from the 7 inhibitor concentrations used in the experiments. Variability in CYP2A6 was assessed by comparing the lowest and highest gene expression (molecule CYP2A6/ng RNA normalized to GAPDH) and activity (rate of metronidazole 2-hydroxylation) among the four donor PHHs. Statistical differences between vehicle control and treatment groups were determined by the Student's t-test (Microsoft Excel 2007, Redmond, WA) or ANOVA (SPSS v. 23, IBM Corp., Armonk, NY) with α=0.05.

Results and Discussion

The inhibitory effects of metronidazole on the biotransformation of nicotine to cotinine are shown in the Dixon plot depicted in Figure 4. Metronidazole inhibited the conversion of nicotine to cotinine competitively (shown by the intersection of lines above

the *x*-axis) with a calculated K_i value of 443.8 ± 93.0 μM in pooled HLMs. The K_i of a compound indicates the potency of the compound as an inhibitor of a particular enzyme, the lower the K_i value, the more potent the inhibitor.

Clinical concentrations of metronidazole seen in human plasma after a 2-gram, one-time oral dose, commonly used to treat *T. vaginalis,* are approximately 300 μM; whereas peak concentrations of metronidazole reached in human plasma after twice daily dosing with 500 mg (e.g., regimens to treat bacterial vaginosis) do not exceed 100 μM (Wang et al., 2011). Utilizing this information, along with knowledge that the concentration of nicotine peaks around 0.3 μM in smokers (Benowitz, Hukkanen, & Jacob, 2009), and our calculated Ki value for metronidazole inhibition of CYP2A6, we calculated the effects on the rate of conversion of nicotine to cotinine using the following modified Michaelis-Menten equation for enzyme inhibition:

$$v = \frac{V_{max} * [S]]}{[S] + K_m (1 + [I]/K_i)}$$

where v is the rate of the reaction, V_{max} is the maximal reaction rate, [S] is the concentration of substrate, K_m is the concentration of substrate where the rate is $V_{max}/2$, and K_i is the inhibitory constant. Following a one-time, 2-g dose of metronidazole, nicotine conversion would be predicted to be maximally inhibited by ~33% early after metronidazole administration. Inhibition would be expected to diminish fairly rapidly and by 12 hours post dose would reach ~20%, which is also the inhibition predicted with twice daily dosing with 500 mg metronidazole thus creating a relatively small decrease in CYP2A6 activity. In one previous study, metronidazole (100 μM) was found to inhibit coumarin 7-hydroxylation in HLMs from a single donor by less than 20% at a substrate concentration equal to Km

(Draper et al., 1997). This single finding is in agreement with our observations. As the objective of that study was to identify potent CYP2A6 inhibitors for *in vitro* studies, the ability of metronidazole to inhibit CYP2A6 activity was not investigated further. Our data suggest that at concentrations achieved with therapeutic doses, metronidazole would likely cause only slight inhibition of CYP2A6-mediated biotransformation of other CYP2A6 substrates *in vivo*.

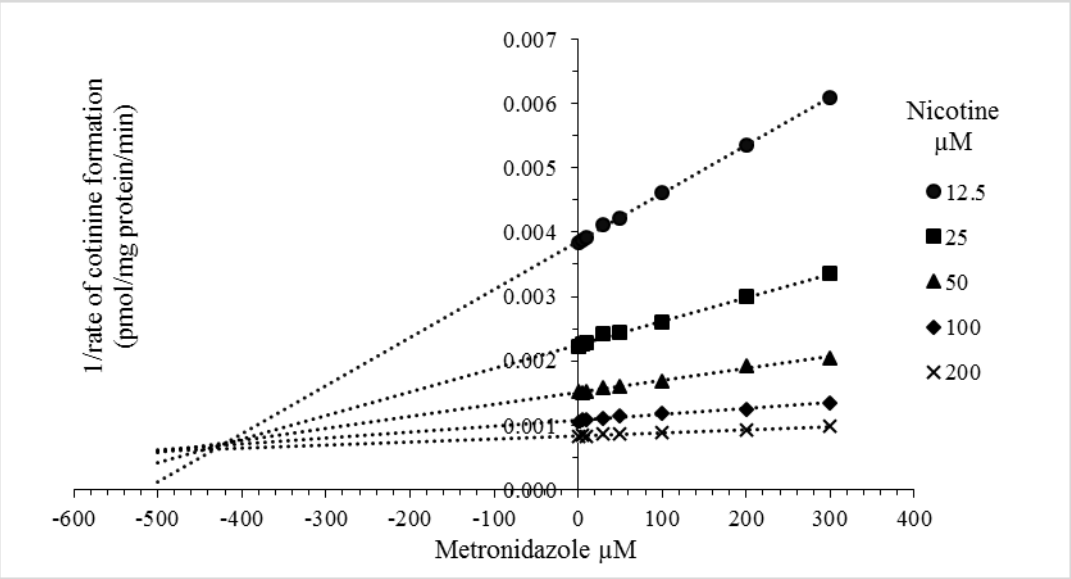

Figure 4. Dixon plot of the effects of various concentrations of metronidazole (0, 5, 10, 30, 50, 100, 200 or 300 µM) on the conversion of nicotine to cotinine. Data are presented as the mean of three separate experiments. Inter-day coefficients of variation were ≤15.5% (range 1.0-15.5%).

The calculated Ki of nicotine (29.5±15.2 µM, Figure 5) obtained in our studies was consistent with previously published Ki values ranging from 4-100 µM in HLMs (Denton, Zhang, & Cashman, 2004; Draper et al., 1997; Li, Li, & Sellers, 1997). The peak plasma concentration of nicotine in smokers is 50 ng/ml which is equivalent to 0.3 µM (Benowitz et al., 2009). Thus, the Ki value of nicotine determined *in vitro* is much higher (nearly 100-fold) than concentrations observed in human plasma and not likely to lead to meaningful inhibition *in vivo.* Studies have evaluated the clearance of nicotine in smokers and noted a reduction over time. Given the Ki of nicotine, other constituents of tobacco smoke such as β-nicotyrine, a product of pyrolysis, have been suggested to cause this effect rather than nicotine itself, yet it is still unclear what is responsible for the observed effect in humans (Denton et al., 2004; Kramlinger et al., 2012). Tranylcypromine served as the positive

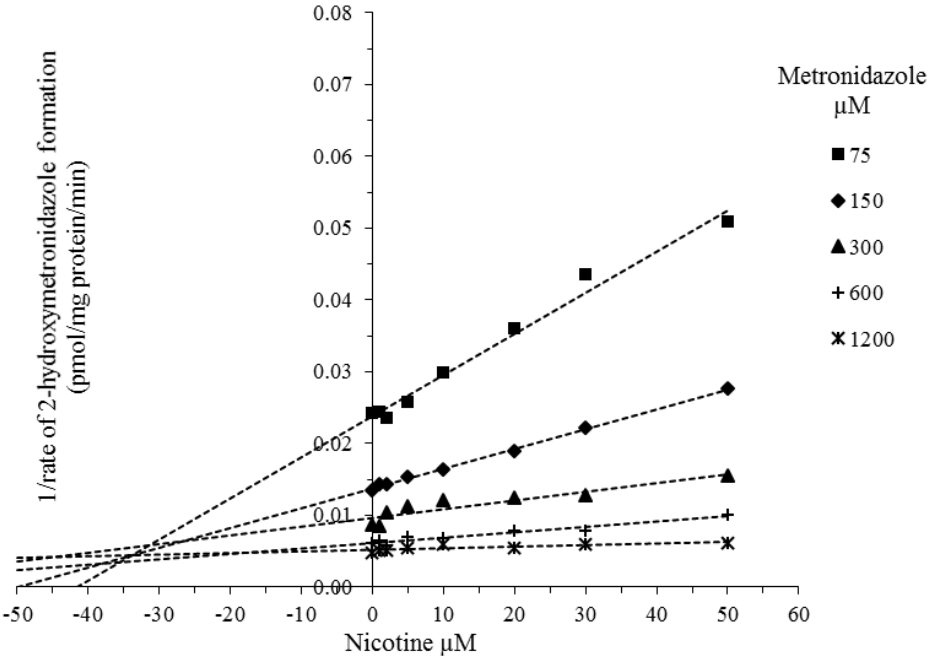

Figure 5. Dixon plot for the determination of the inhibitory constant (Ki) of nicotine. r, rate in pmol/mg/min; concentrations of inhibitors are listed in µM for nicotine (positive control). Data are representative of three separate experiments on three separate days, %CV ranged 1.9-22.6 among the nine inhibitor concentrations.

control for the inhibition experiments and demonstrated a Ki value (0.2 μM) consistent with prior reports in HLMs (Khojasteh, Prabhu, Kenny, Halladay, & Lu, 2011; Stephens et al., 2012).

The capacity for metronidazole and nicotine to act as an inducer of CYP2A6 was assessed in PHHs. Pooled PHHs were utilized to optimize experimental conditions related to the determination of *CYP2A6* gene expression and activity. Data in Figure 6 illustrate significant induction of CYP2A6 mRNA expression and activity by the positive control in pooled PHHs as expected. Nicotine at a concentration greater than three times the peak plasma concentrations seen in smokers [1 μM] failed to induce CYP2A6 mRNA or activity in pooled PHHs. Nicotine is a ligand of pregnane X receptor (PXR), which is a known CYP2A6 regulatory pathway leading to induction; however, this has only been shown at high concentrations (≥1 mM) not relevant to those seen in human plasma (Itoh et al., 2006; Lamba et al., 2004).

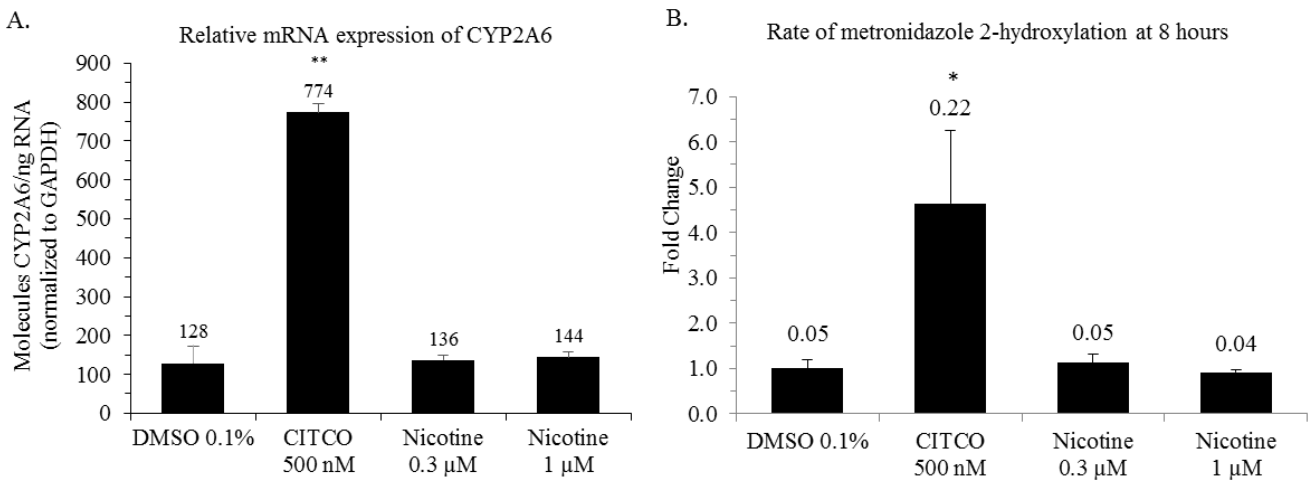

Figure 6. Effects of nicotine treatment on CYP2A6 expression and activity in pooled primary human hepatocytes (PHHs). A 10-donor pool of PHHs (n=3 replicates/treatment group) was treated with vehicle (DMSO 0.1%), positive control (CITCO 500 nM), or nicotine (0.3 µM and 1 µM) for 72 hours. Significance was determined by comparing values from treatment groups with vehicle treated control PHHs. Panel A shows expression levels of CYP2A6 mRNA as determined via qRT-PCR and normalized to GAPDH. Panel B shows the effects of test compound treatment on the conversion of metronidazole to 2-hydroxymetronidazole, a marker reaction for CYP2A6 activity. Corresponding rates of metronidazole 2-hydroxylation are listed above each bar. Data for the 8-hour time points are shown, reflecting maximum conversion of metronidazole to its metabolite. Error bars indicate standard deviation from the mean. *P<0.05; **P<0.01.

To characterize the effect of high dose metronidazole, equivalent to peak plasma concentrations of 300 µM, on CYP2A6 mRNA and activity and understand inter-individual variability, experiments were undertaken in individual donor PHHs. Although treatment with the positive control, CITCO, induced CYP2A6 mRNA expression and activity between 2.6- to 5.8-fold and 2.5- to 4.2-fold, respectively, in individual PHHs, with one exception, metronidazole treatment had little or no effect on CYP2A6 mRNA expression or activity, as shown in Figure 7. An increased rate of 2-hydroxymetronidazole formation over that of control was observed in PHHs treated with metronidazole from one donor (HUM4038). Metronidazole treatment of PHHs from this donor led to a statistically significant 1.4-fold increase in 2-hydroxymetronidazole formation. However, the extent of this increase does not

meet the criteria set forth by the FDA for induction. The 2017 draft guidance from the FDA recommends reporting a positive finding of induction if mRNA levels increase ≥ 2-fold that of controls and activity is greater than 20% of the positive control increase over baseline (control) (U.S. Department of Health and Human Services, 2017). This suggests that at therapeutic concentrations achieved *in vivo*, metronidazole is unlikely to cause induction of CYP2A6 clinically.

As depicted in Figure 7, considerable inter-individual variability in both gene expression and activity was observed among PHHs from the various donors. After treatment with vehicle control, *CYP2A6* gene expression varied by 36.1-fold and activity measured by the conversion of metronidazole to 2-hydroxymetronidazole varied by 21.4-fold. The extent of this observed variability is consistent with the variability in CYP2A6 activity observed in previous studies utilizing HLMs. Tanner et al. (2017) demonstrated that CYP2A6 activity, as measured by nicotine c-oxidation and coumarin 7-hydroxylation, varied 275-fold and 225-fold, respectively, among a panel of more than 300 HLM preparations. In another study, HLMs obtained from 16 donors demonstrated over 10-fold variability in rates of 2-hydroxymetronidazole formation among samples after treatment with metronidazole equivalent to therapeutic plasma concentrations (Pearce et al., 2013)

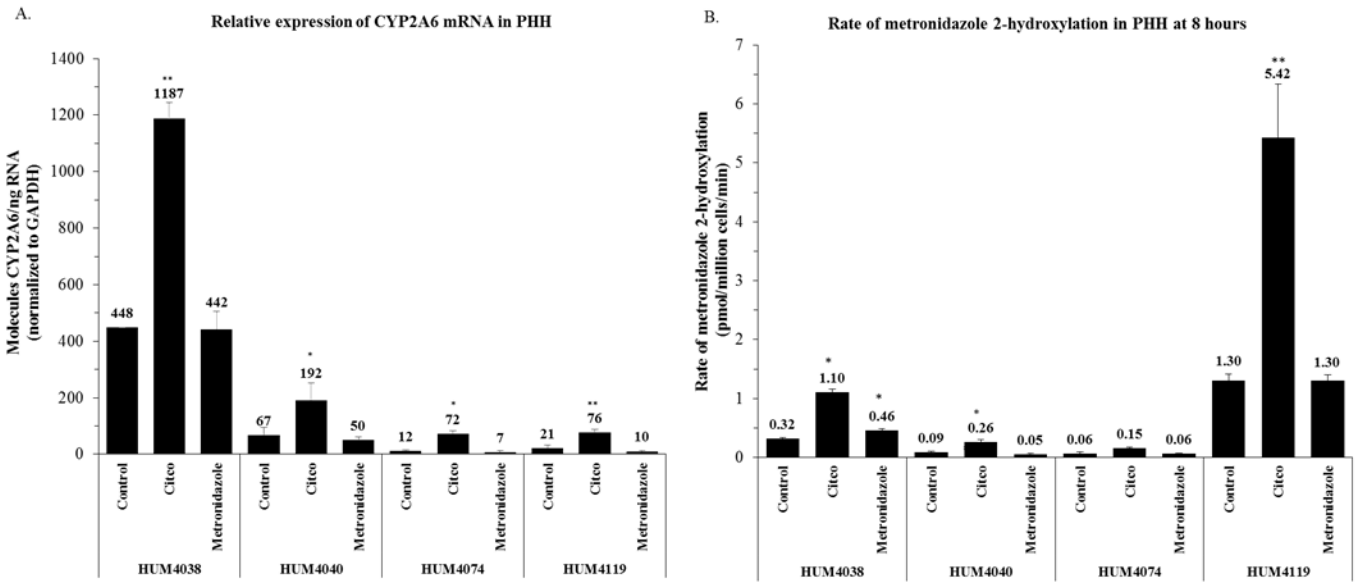

Figure 7. Effects of metronidazole treatment on CYP2A6 expression and activity in primary human hepatocytes (PHHs). PHHs from four female donors (n=3 replicates/treatment group) were treated with vehicle (DMSO 0.1%), positive control (CITCO 500 nM), or metronidazole (300 μM) for 72 hours. Panel A shows expression levels of CYP2A6 mRNA as determined via qRT-PCR and normalized to GAPDH. Panel B shows the effects of test compound treatment on the conversion of metronidazole to 2-hydroxymetronidazole, a marker reaction for CYP2A6 activity. Data for the 8-hour time points are shown, reflecting maximum conversion of metronidazole to its metabolite. In both data sets, significance was determined by comparing values from treatment groups with vehicle treated control PHHs (*P<0.05; **P<0.01). Error bars indicate standard deviation from the mean.

One potential limitation to this study was that the activity assay used to assess the induction potential of metronidazole treatment on CYP2A6 in PHHs involved the measurement of the product, 2-hydroxymetronidazole, from the substrate, metronidazole, administered *in situ*. However, the issues associated with this choice are likely to be minimal. The likelihood of carry-over of metronidazole from the treatment phase to the determination of *in situ* activity is low since the levels of 2-hydroxymetronidazole were below the limits of detection for both controls and PHHs treated with metronidazole at the 30-minute time point. In addition, we noted that the rates of metronidazole 2-hydroxylation did not markedly differ between controls and metronidazole-treated PHHs at the various

time points examined beyond two hours (results shown for only the 8-hour time point in Figure 7).

In conclusion, this is the first investigation to characterize the effects of metronidazole and provides corroboratory data regarding the behavior of nicotine on CYP2A6 activity and expression. Our observations suggest that metronidazole and nicotine are not potent inhibitors or inducers of CYP2A6 *in vitro*. We speculate that when administered at therapeutic doses, metronidazole is unlikely to cause induction of CYP2A6 *in vivo*, and should cause no more than a slight inhibition of CYP2A6 activity in patients. In addition, nicotine is not predicted to be an inhibitor or inducer of CYP2A6 at concentrations relevant to those expected in humans. Further studies in humans would be important to confirm these predictions.

CHAPTER 3

DEVELOPMENT OF A UPLC-MS/MS METHOD FOR QUANTITATION OF METRONIDAZOLE AND 2-HYDROXY METRONIDAZOLE IN HUMAN PLASMA AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

Adapted from the manuscript titled, Development of a UPLC-MS/MS method for quantitation of metronidazole and 2-hydroxy metronidazole in human plasma and its application to a pharmacokinetic study
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Introduction

Metronidazole is an antimicrobial with a unique spectrum of activity against both anaerobic bacteria and parasites (Samuelson, 1999). It has a variety of therapeutic uses across the lifespan to treat infections caused by *C. difficile, T. vaginalis*, *G. vaginalis*, *H. pylori,* as well as the treatment of sepsis in premature infants (Clark, Bloom, Spitzer, & Gerstmann, 2006; Cudmore, Delgaty, Hayward-McClelland, Petrin, & Garber, 2004; Ralph, 1983; Rosenblatt & Edson, 1987). Daily doses range from 15 mg/kg in infants to 2000 mg in adults. Metronidazole undergoes biotransformation to its primary metabolite, 2-hydroxymetronidazole, a reaction selectively catalyzed by CYP2A6 (Pearce et al., 2013). 2-Hydroxymetronidazole maintains approximately 65% of the antimicrobial activity of metronidazole and has been reported to have a longer half-life (Cudmore et al., 2004; Pendland, Piscitelli, Schreckenberger, & Danziger, 1994; Ralph, 1983; Rosenblatt & Edson, 1987). The pharmacokinetics (PK) of metronidazole continue to be studied in different clinical applications, one of which is to refine dosing recommendations, particularly in neonates and young children (Asín-Prieto et al., 2015; Dannelley, Martin, Chaaban, &

Miller, 2017; de C. Bergamaschi et al., 2014; Sakurai et al., 2016; Wang et al., 2017). Another application for the pharmacokinetic data from the quantitation of metronidazole and its major metabolite is the potential for CYP2A6 activity phenotyping, which could provide insight into the variability within this pathway of drug metabolism.

Validated analytical methods exist to measure concentrations of metronidazole in human plasma; however, only a few have also sought to quantitate 2-hydroxymetronidazole. Methods utilizing high performance liquid chromatography with ultraviolet detection (UHPLC-UV) to quantify both metronidazole and 2-hydroxymetronidazole have been described (Houghton, Hundt, Muller, & Templeton, 1982; Nilsson-Ehle, Ursing, & Nilsson-Ehle, 1981; Suyagh et al., 2011; Wheeler, De Meo, Halula, George, & Heseltine, 1978), but demonstrate reduced sensitivity (lower limits of quantitation ranging between 0.8 – 3.5 μM) and/or require large volumes of human plasma (up to 5 mL) to achieve the desired sensitivity. One sensitive HPLC-UV assay (lower limit of quantitation of 50 nM) was developed by de C. Bergamashi et al. (de C. Bergamaschi et al., 2014), but this method still required 500 mL of plasma and also did not quantify 2-hydroxymetronidazole. Two sensitive LC-MS/MS analytical assays for the quantitation of metronidazole in human plasma were developed by Silva et al. (Silva et al., 2009) and Cohen-Wolkowiez et al. (Cohen-Wolkowiez, White, Bridges, Benjamin, & Kashuba, 2011) with lower limits of quantitation of 0.3 μM. The method described by Cohen-Wolkowiez et al. requires only 50 μL of plasma, whereas the method of Silva et al. requires 250 μL of plasma and contains complex sample preparation steps. Unfortunately, neither of these methods quantified 2-hydroxymetronidazole concentrations. Quantitation of both metronidazole and 2-

hydroxymetronidazole concentrations are necessary to determine for the catalytic activity of CYP2A6 *in vivo.*

Currently there are no validated, high throughput, sensitive methods for the quantitation of metronidazole and 2-hydroxymetronidazole levels in human plasma. Methods that require low volumes of plasma (<50 μL) might be attractive for studies with patient populations where cumulative sample volume over a pharmacokinetic time course is a limitation. Therefore, the purpose of this work was to develop and validate a rapid and sensitive UPLC-MS/MS method for the simultaneous determination of metronidazole and 2-hydroxymetronidaole in human plasma using low volumes of human plasma (10 μL) for application in a clinical study designed to evaluate the use of metronidazole as an *in vivo* probe for CYP2A6 activity.

Materials and Methods

Chemicals and Reagents

Metronidazole was obtained from Sigma-Aldrich (St. Louis, MO). 2-Hydroxymetronidazole, metronidazole-d4, and 2-hydroxymetronidazole-d4 were purchased from Toronto Research Chemicals (North York, ON). Optima grade methanol and formic acid were acquired from Fisher Scientific (Fair Lawn, NJ). Ultrapure water was produced by a Barnstead Nanopure column system (Thermo Scientific, Philadelphia, PA). All other chemicals were of analytical grade. Blank human plasma from six individual donors was prepared from scavenged blood samples at Children's Mercy Kansas City and used to determine recovery and selectivity. Six lots of pooled human plasma were purchased from Bioreclamation Inc. (Hicksville, NY) and subsequently combined to create a diverse pool

with sufficient volume for the preparation of calibration standards and quality control samples throughout the study.

Instrumentation and Analytical Conditions

Chromatography was performed using a Waters Acquity UPLC system with an Acquity BEH C18 column (1.7 μm, 2.1 x 100 mm, Waters, Milford, MA) and Acquity BEH C18 Vanguard Pre-Column (1.7 μm, 2.1 x 5 mm). Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in methanol. Table 4 lists the LC linear gradient profile utilized. The column temperature was maintained at 40°C; the sample storage compartment was maintained at 4°C. Under these conditions 2-hydroxymetronidazole and metronidazole eluted at 3.2 and 3.5 minutes, respectively. The total run time was 8 minutes.

Table 4

UPLC Gradient

Time (min)	% Mobile phase A	% Mobile phase B	Flow rate (μL/min)
Initial	99	1	250
0.1	99	1	250
4.1	1	99	250
5.1	1	99	250
5.2	99	1	250
8	99	1	250

Mass spectrometric detection was performed on a Waters TQD tandem mass spectrometer equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode and quantitation was performed using the following transitions: *m/z* 171.85 > 127.9 for metronidazole, *m/z* 175.85 > 127.9

for metronidazole-d4, *m/z* 187.9 > 125.9 for 2-hydroxymetronidazole, and *m/z* 191.9 > 125.9 for 2-hydroxymetronidazole-d4. For all transitions, a dwell time of 50 ms, cone voltage of 24 V and a collision energy of 16 eV was used. The capillary voltage was set at 2.97 kV, and the source temperature was maintained at 400 °C with a gas flow of 800L/h. Cone gas flow was maintained at 10 L/h. Argon served as the collision gas. Data acquisition and instrument control were performed with MassLynx software version 4.1, and quantitation was accomplished using TargetLynx (Waters, Milford, MA).

Standard Solution and Quality Control Sample Preparation

Calibration standard spiking solutions (1, 3, 10, 30, 100, 300, 1000, 3000 μM) were prepared by combining metronidazole and 2-hydroxymetronidazole in appropriate dilution with water from a 1 mg/ml aqueous standard stock solution. Quality control (QC) spiking solutions (1, 2.5, 37.5, 2500 μM) were made in a similar fashion. Calibration standards and QCs were prepared by aliquoting 10 μL of stock spiking solutions in 90 μL plasma. An internal standard (IS) spiking solution (10 μM) containing metronidazole-d_4 and 2-hydroxymetronidazole-d_4 was prepared by combining the two analytes with appropriate dilution. A working IS solution was created by 10-fold dilution of IS spiking solution with 100 mM ammonium bicarbonate, pH 7.0 and 100 μL were added to each sample. All stock and spiking solutions were stored at -20°C.

Sample Preparation

Plasma samples were stored in a -80°C freezer prior to analysis. Samples were thawed on ice prior to processing. An aliquot of plasma (10 μL) was diluted in 90 uL of H_2O and mixed with an equal volume (100 uL) of IS working solution in 1.5 mL microcentrifuge tubes. Samples were gently mixed using a bench top Eppendorf MixMate® at 2000 rpm for

2 minutes, followed by centrifugation at 15,600 *g* for 5 minutes to remove any particulates. The samples were then purified by solid phase extraction (SPE).

Each well of Oasis HLB 30 μm, 96-well extraction plates (Waters, Milford, MA, USA) was conditioned and equilibrated with 200 μL methanol followed by 200 μL 100 mM Ammonium Bicarbonate buffer, pH 7.0. Buffered sample (200 μL) was loaded in each well, vacuum applied, and eluted slowly through each column. Wells were washed with 100 mM ammonium bicarbonate, pH 7.0 (200 μL). The analytes were then eluted with methanol (500 μL) by slow vacuum in a 96-well collection plate followed by dilution with 500 μL deionized H_2O, and mixed thoroughly by gentle pipetting. The collection plate was covered with a silicon mat and 3 μL from each well was injected into the UPLC-MS/MS.

Method Validation

Method validation was based on the United States Food and Drug Administration document "Guidance for Industry: Bioanalytical Method Validation" dated May 2001 (U.S. Department of Health and Human Services, 2001).

Selectivity. To determine if samples might contain potential interfering substances, blank biological matrix should be tested for selectivity of the method. Blank plasma samples obtained from six different individuals were analyzed for the presence of interfering peaks in "blank samples" (matrix only, no IS or analyte), "zero samples" (matrix only with IS), and samples spiked with standard at the lower limit of quantitation (LLOQ) (matrix with IS and 0.1 μM of both analytes). Any observed co-eluting peak should have an area <20% of the area of the analytes.

Recovery and matrix effects. The recovery experiments were performed at the low QC (0.25 μM) and high QC (250 μM) standard concentrations using six replicates of each

concentration in blank plasma obtained from six different individuals. The recovery of metronidazole and 2-hydroxymetronidazole was determined by comparing analytes of pre-extraction spiked samples to plasma samples that were spiked with analytes following extraction by SPE. The matrix effect (%) was evaluated by comparing the same concentrations of post-extraction spiked plasma samples to post-extraction spiked aqueous reference samples (H_2O:100 mM ammonium bicarbonate, pH 7.0, 50:50, v/v), performed in triplicate. Concentrations were calculated using IS-corrected (analyte:IS ratio) peak areas for each analyte. Coefficients of variation (%CV) were determined and a deviation of ≤15% was considered acceptable.

Accuracy, precision, linearity and dilution integrity. Intra-day and inter-day precision and accuracy were determined in pooled plasma (n=6) at four different concentrations: LLOQ, low QC (approximately 3xLLOQ), mid QC, and high QC (approximately 80% upper limit of quantitation), repeated on three separate days. Precision is expressed as the coefficient of variation (%CV) of the repeated measurements. Accuracy is expressed as the (measured analyte concentration/target analyte concentration) x 100. Calibration curves were constructed by plotting the ratio of analyte peak area to IS peak area versus nominal analyte concentration using least squares regression with a 1/x weighting factor. Dilution integrity was assessed by spiking pooled blank human plasma with 3000 μM metronidazole or 2-hydroxymetronidazole and diluting 1:10 with pooled blank human plasma and back-calculating the concentration. All samples were prepared in triplicate. Acceptability criteria for accuracy and precision was set at ±15% deviation for all samples except for the LLOQ concentration which was allowed ±20% deviation based on FDA guidance documents (U.S. Department of Health and Human Services, 2001).

Stability. The long term stability of analytes in human plasma was examined at low QC (0.25 μM) and high QC (250 μM) concentrations in three replicates of pooled human plasma and stored for 1 month and 3 months at -80°C and -20°C. Short-term stability was examined after 4 hours at room temperature and also after 4 hours of storage at 4°C. Samples were then thawed on ice prior to processing. The freeze-thaw stability of analytes was determined at low QC (0.25 μM) and high QC (250 μM) concentrations in three replicates of pooled plasma. Freeze-thaw samples were stored at -80°C and then allowed to thaw at room temperature for three repetitive freeze-thaw cycles spaced 24 hours apart. Auto-sampler stability was assessed by reinjection of processed samples which were allowed to remain in a cooled auto-sampler (4°C) for up to 48 hours after initial injection. To be considered stable, samples had to meet acceptable limits for accuracy and precision of the nominal concentration ±15%.

Pharmacokinetic Application

The analytical method was used to determine concentrations of analytes in healthy, non-pregnant adult volunteers aged 18-56 years with BMI 18.5-29.9 given metronidazole (500 mg) after an overnight fast. Participants were deemed "healthy" after undergoing a medical history, physical exam, and laboratory evaluation (chemistry panel, complete blood count, hepatic function panel, and urine pregnancy, for females of child-bearing potential). Participants abstained from taking any prescription or over-the counter-medications, using tobacco, and consuming caffeine, alcohol, or grapefruit juice prior to the study. Three milliliters of blood were drawn from a peripheral intravenous catheter (PIV) prior to ingestion of metronidazole (0 h) and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, 20, 24, 48 h after ingestion in a lavender EDTA tube, gently mixed by inversion, and then subjected to

centrifugation at 1000 *g* for 10 minutes at 4°C to allow for separation of plasma. Plasma was aliquoted into individual cryovials and stored at -80 °C until analysis. All study participants provided informed consent and the study was approved by the Institutional Review Board at Children's Mercy Kansas City.

Following determination of analyte concentrations, compartmental pharmacokinetic analyses were performed using Kinetica 5.0 (Thermo Scientific, Philadelphia, PA) to determine rate constants for elimination and distribution. Half-lives were calculated using the following equations: $t_{1/2\ elim} = 0.693/\lambda_z$ and $t_{1/2\ form} = 0.693/k\alpha$. The apparent terminal elimination rate constant (λ_z) and formation rate constant (α) were determined from linear least squares regression of log-linear plasma concentration versus time curves. Each curve was assessed for goodness of fit based on mathematical criteria (objective function, Akaike, Schwartz). Coefficients of variation (%CV <30%) was used to determine parameter estimates.

Results and Discussion

Method Development

In order to assess the ability of metronidazole to serve as an *in vivo* probe for CYP2A6 activity, we first needed a sensitive and robust assay that could quantitate low concentrations of both metronidazole and its main metabolite, 2-hydroxymetronidazole, in human plasma. A method that requires low volumes of plasma (<50 μL) and is amenable to high throughput was also seen as highly desirable. As there were no methods that met these criteria when we began our project, we developed and validated the assay described here.

Plasma is a complex matrix containing salts, lipids, proteins and other small organic compounds, substances that may alter the resolution and/or ionization of analytes. These co-

eluting endogenous compounds may suppress or enhance ion formation in ESI sources, effecting the accuracy of quantitation (Trufelli et al., 2011). Ion enhancement (24.3–38.7%) has been reported in equine plasma samples containing low concentrations (75 ng/mL) of metronidazole (Ilomuanya et al., 2015) following liquid-liquid extraction, so the importance of sample clean-up in our development process cannot be over-stated. Protein precipitation methods have been used in previous metronidazole assays of human plasma samples (Trufelli et al., 2011), but protein precipitation only removes a portion of the protein present and does not remove phospholipids or salts, which are known to create matrix effects. In our lab, we have tried protein precipitation in numerous projects and, while they often work for analyses ~300 samples, we have found declining performance of the method over-time due to the build-up of phospholipids in the analytical column. To create a more robust and reproducible method with higher throughput, in our experience rigorous sample clean-up has been worth the up-front effort.

Other metronidazole assays of plasma samples have utilized liquid-liquid extraction (LLE) techniques (Cohen-Wolkowiez et al., 2011; Ilomuanya et al., 2015; Gatchev, Bräter, & de Mey, 2006). LLE can yield clean extracts, but these methods are often cumbersome, can involve analyte losses and often require relatively large solvent volumes. Solid phase extraction (SPE) techniques can also yield clean samples and have the advantage that samples may be scaled down to sample volumes below 50 µL with the potential for automating the procedure. The cost per sample, though, is typically higher using SPE versus LLE. We opted to develop the method described here utilizing solid-phase extraction plates to clean up our samples, in large part due to the potential for low sample volume requirements and the possibility of automating the procedure in the future.

Several types of 96-well SPE plates that were available in our laboratory were examined for their ability to minimize matrix effects and maximize recovery of metronidazole and 2-hydroxymetronidazole in human plasma. SPE plates designed to principally remove phospholipids from samples (Phenomenex Phree and Supelco Hybrid SPE removal) were observed to have low matrix effects (86-102%) but rather poor recoveries of metronidazole and 2-hydroxymetronidazole (67 and 78%; 61 and 75% for the Phree and Hybrid SPE plates, respectively). Because metronidazole has both polar and weak basic characteristics, we next chose to examine a mixed-mode cation exchange plate (MCX; Waters). Mixed mode SPE utilizes two retention mechanisms; one based on reversed-phase chemistry, the other based on ion exchange chemistry, coupled to extract analytes from complex matrices using the same SPE column. The MCX plate led to 70% recovery of metronidazole, but the metabolite was not well retained using the manufacturer's recommended sample buffer (4% phosphoric acid). Fifteen percent of 2-hydroxymetronidazole was lost by lack of capture by the column with first application (i.e., flow-through loss). Subsequently, 15% was lost during the 2% formic acid wash and 40% was lost during the 100% methanol wash. A change of buffer to trifluoroacetic acid (TFA) was done in an attempt to acidify the sample, however, this yielded almost complete loss of both analytes in the flow-through eluate (i.e., lack of retention on the MCX column). Hence, the MCX plate was abandoned. Finally, a hydrophilic-lipophilic-balanced plate (Oasis HLB; Waters) was examined. The Oasis HLB sorbent contains equal amounts of hydrophilic and lipophilic monomers and provides reversed-phase capability with enhanced capture of polar analytes (Arsenault, 2012). The Oasis HLB plate demonstrated superior recovery of both metronidazole and its polar metabolite, 2-hydroxymetronidazole, with limited matrix effects

(Table 6). Therefore, the Oasis HLB plate was judged to provide superior sample clean-up and was used for the method validation experiments.

Because the eluate from the Oasis HLB plate was largely composed of methanol, two hydrophilic interaction chromatography (HILIC) columns were investigated to see if the eluates might be injected directly into the UPLC-MS/MS system without further sample dilution or evaporation followed by reconstitution. Hydrophilic interaction chromatography has an elution pattern similar to normal phase chromatography, however, a reversed-phase solvent system is used which overcomes some of the obstacles of normal phase chromatography, like limited analyte solubility (Grumbach, Fountain, 2010). Although symmetrical peaks and satisfactory signals were obtained with a HILIC BEH 2.1 x 50 mm column (mobile phase – A: 100 mM ammonium formate, pH 3.0 and B: 0.1% formic acid-methanol) and a BEH Amide 1.0 x 50 mm column (mobile phase – A: 100 mM ammonium formate, pH 3.0 and B: 0.1% formic acid-methanol), both columns displayed poor retention of metronidazole and 2-hydroxymetronidazole and further efforts using HILIC columns for this project were abandoned.

Several other investigators have successfully used reversed-phase chromatography (in particular C-18 columns) coupled with mass spectrometric detection of metronidazole (Klimowicz, Bielecka-Grzela, Tomaszewska, 2002; Vanol et al, 2018; Jeffery et al, 2017), hence, our subsequent efforts examined the performance of several reversed-phase columns that were available in the laboratory. The following columns were tested using combinations of 0.1% formic acid in methanol or acetonitrile as the organic phase and 0.1% formic acid in water or 10 mM ammonium acetate, pH 5.0, as the aqueous phase: a Zorbax SB Aq (1.8 μm,

2.1 x 100 mm), which excels at separating polar metabolites, a Cortecs C-18 (1.6 µm, 2.1 x 100 mm) and an Acquity UPLC BEH C-18 (1.7 µm, 2.1 x 100 mm). Although peak resolution and sensitivity were adequate with all three columns, the retention of the analytes by the Zorbax and Cortecs columns was poor and thus, were deemed inadequate. The best chromatographic conditions were achieved with the Acquity BEH C-18 column using mobile phases consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) using the linear gradient described in the Methods section above.

Table 5

Recovery Determined in Six Different Lots of Plasma (n = 6, technical triplicates)

Concentration	Analyte	Recovery (%)
Low (0.25 µM)	Metronidazole	99.1 ± 15.4
Low (0.25 µM)	2-Hydroxymetronidazole	86.1 ± 11.4
High (250 µM)	Metronidazole	88.0 ± 6.6
High (250 µM)	2-Hydroxymetronidazole	77.7 ± 5.2

Table 6

Matrix Effect Determined in Pooled Plasma (n = 3)

Concentration	Analyte	Matrix Effect (%)
Low (0.25 μM)	Metronidazole	105.2 ± 0.01
Low (0.25 μM)	2-Hydroxymetronidazole	106.1 ± 0.01
High (250 μM)	Metronidazole	101.6 ± 2.3
High (250 μM)	2-Hydroxymetronidazole	99.4 ± 0.9

Method Validation

Selectivity. No inherent interference was observed at the retention times and MRMs for metronidazole, metronidazole-d_4, 2-hydroxymetronidazole and 2-hydroxymetronidazole-d_4. Figure 8 illustrates representative chromatograms of blank donor plasma (panels A-D), plasma spiked with LLOQ (panels E-H) and a plasma sample from a patient at 48 hours after receiving an oral dose of 500 mg metronidazole (panels I-L).

Recovery and matrix effects. The recovery of analytes from a 10 μL aliquot of plasma was determined at the LQC and HQC concentrations (see Table 5). Mean recovery of both analytes was >77% at both concentrations evaluated. Matrix effects were minimal (ranging from 99.4-106.1%, Table 6) in pooled plasma.

Accuracy, precision, linearity and limits of detection. The results listed in Table 7 provide a summary of the accuracy and precision of the method determined at four concentrations (LLOQ, LQC, MQC and HQC) of each analyte spiked in plasma. At the LLOQ the accuracy was within 84.4-91.7 % and the precision was ≤16.1%. For the other QC concentrations, accuracy was noted to be within 87.3-108.5% and the precision was

≤13.1%. Dilution integrity samples had an average measured concentration (corrected for dilution factor) of metronidazole and 2-hydroxymetronidazole that was 98.8 and 101.5%, respectively, of reference standard concentrations with precisions <5.6%, demonstrating that plasma samples with metronidazole or 2-hydroxymetronidazole concentrations up to 3000 μM can be accurately quantitated with a 10-fold dilution. The LLOQ concentrations achieved in this study are approximately 3-fold lower than the lowest quantifiable concentrations reported to date (Cohen-Wolkowiez et al., 2013). Calibration curves showed good, reliable linearity over the concentration range of 0.1-300 μM for both analytes in plasma ($r \geq 0.999$).

Table 7

Intra- and Inter-day Accuracy, Precision, and Dilution Integrity

Target conc (μM)	Intra-day (n=6)			Inter-day (n=3)		
	Measured conc (μM)	Accuracy (%)	Precision (%CV)	Measure conc (μM)	Accuracy (%)	Precision (%CV)
Metronidazole						
LLOQ (0.1)	0.09	88.3	11.1	0.08	84.4	5.0
LQC (0.25)	0.25	98.7	9.5	0.25	99.1	0.8
MQC (3.5)	3.67	104.8	8.5	3.66	104.5	0.7
HQC (300)	271.36	108.5	11.5	259.16	103.7	10
Dilution QC (3000; 1:10)	2963.13	98.8	4.7			
2-hydroxymetro-nidazole						
LLOQ (0.1)	0.09	91.7	16.1	0.09	87.8	7.7
LQC (0.25)	0.22	87.3	11.7	0.23	90.7	2.5
MQC (3.5)	3.52	100.6	8.8	3.52	100.6	0.2
HQC (300)	271.04	108.4	13.1	258.46	103.4	10.8
Dilution QC (3000; 1:10)	3044.42	101.5	5.6			

Stability. Stability was assessed by testing concentrations of QC samples at a variety of storage conditions (see Table 8). Sample extracts were stable on the auto-sampler for at least 48 hours with bias of <6% for concentrations of metronidazole and 2-hydroxymetronidazole ranging between 0.3-300 μM and <14.1% for the LLOQ concentration (0.1 μM). Both analytes were stable when stored at -80°C for up to 3 months. Although 2-hydroxymetronidazole was stable when stored at -20°C for up to 3 months, metronidazole displayed an 18.2% bias when stored under these conditions for up to 3 months, which exceeds the acceptability criteria (nominal±15%).

Table 8

Stability

	Metronidazole		2-hydroxymetronidazole	
	LQC (%bias)	HQC (%bias)	LQC (%bias)	HQC (%bias)
Room temp, 4 h	1.3	-14.1	7.8	-15.2
4°C, 4 h	3.1	-5.7	12.2	-5.7
Freeze/thaw (3 cycles)	-0.6	-0.9	14.0	-2.3
-20°C, 1 month	-1.9	-6.3	-8.5	-5.3
-20°C, 3 month	18.2	0.4	-3.5	-3.2
-80°C, 1 month	6.9	-8.7	1.9	-6.4
-80°C, 3 month	-0.6	-2.7	-7.3	-2.7

Therefore, it is prudent to store plasma samples containing metronidazole and/or 2-hydroxymetronidazole at -80°C if long-term storage is anticipated prior to assay. The clinical samples for the pharmacokinetic study were stored at -80°C until analyses were completed.

Pharmacokinetic Application

The analytical procedure was used to determine plasma concentrations of analytes in six healthy volunteers participating in a larger clinical pharmacokinetic study. Plasma concentration-time curves for six participants are presented in Figure 9 and determined pharmacokinetic parameters are listed in Table 9. Metronidazole concentrations were within the range of calibration standards for all samples analyzed. Plasma concentrations of 2-hydroxymetronidazole were within the range of calibration standards with the following exceptions: the concentration of 2-hydroxymetronidazole was below the calibration range in 4 patients at the 0.25 h time point and in 1 patient at the 0.5 h time point.

Table 9

Pharmacokinetic Parameters of Metronidazole and 2-Hydroxymetronidazole in Human Plasma after Oral Administration of 500 mg Metronidazole (mean ± SD, n=6)

	Metronidazole	2-hydroxymetronidazole
C_{max} (μM)	63.7 ± 19.4	8.2 ± 3.5
T_{max} (h)	1.4 ± 1.0	12.3 ± 4.8
$AUC_{0\text{-}48h}$ (μM*h)	740.7 ± 172.5	226.8 ± 73.3
$AUC_{0\text{-}inf}$ (μM*h)	765.7 ± 197.2	253.4 ± 72.6
$T_{1/2\ elimination}$ (h)	8.9 ± 3.5	3.3 ± 1.4
$T_{1/2\ formation}$ (h)	n/a	16.4 ± 3.3

Values that fell below the LLOQ concentration were assigned a value of half the LLOQ concentration (0.05 μM) after reviewing each back-calculated value and chromatogram to ensure adequate peak resolution. Concentrations of metronidazole and 2-hydroxymetronidazole determined by this assay are comparable to concentrations previously reported after similar oral doses (Gatchev, Bräter, & de Mey, 2006; Jensen & Gugler, 1983; Jessa, Goddard, Barrett, Shaw, & Spiller, 1997; Loft et al., 1986). There are some conflicting reports regarding the half-life of 2-hydroxymetronidazole. The mean half-life seen in our cohort of healthy adults was 13.1 ± 3.7 h and ranged from 9.1-20.4 h. Jessa et al reported a "true" 2-hydroxymetronidazole half-life of 3.6 ± 0.7 h (Jessa et al., 1997). Others reported half-lives of elimination for the hydroxy-metabolite ranging from 10.8 – 18 hours (Houghton et al., 1982; Gatchev, Bräter, & de Mey, 2006; Loft et al., 1986; Bergan, Thorsteinsson, 1986). Discrimination of the formation half-life from the elimination half-life was absent from these latter publications. Ongoing work is being done by our group to more fully characterization formation and elimination of the metabolite. 2-Hydroxymetronidazole has more than half the antimicrobial activity of the parent compound and is thought to provide additive growth inhibition towards aerobes and anaerobes (Jones, Geary, Alawattegama, Kinghorn, & Duerden, 1985; Pendland et al., 1994; Ralph & Amatnieks, 1980; Rosenblatt & Edson, 1987). Therefore, a more complete understanding of metabolite kinetics may provide better information to clinicians and scientists who strive to implement precision therapeutics. As expected, marked variability of 7.7-fold and 4.9-fold was observed in exposure to metronidazole and 2-hydroxymetronidazole, respectively, among the subjects. It is likely that a number of factors contribute to the pharmacokinetic variability among participants. Endogenous compounds like estrogen are proposed to contribute to

CYP2A6 activity through nuclear receptor modulation (Higashi et al., 2007). Each individual's CYP2A6 genotype is likely an important variable as evidence suggests the ability to cluster activity based on genotype-suggested phenotype for other CYP2A6 substrates (e.g., extensive versus slow metabolizers) (Zhou et al., 2017). Also of note, metronidazole was not dosed on a weight adjusted basis, although impact of weight as a variable was mitigated by recruiting only healthy volunteers with a BMI 18.5-29.9 (non-obese, non-underweight) to participate in the clinical study.

Conclusions

A sensitive, high-throughput UPLC-MS/MS method with simple, rapid sample preparation which uses only 10 μL of human plasma was developed and validated for the quantitation of metronidazole and its primary metabolite, 2-hydroxymetronidazole, according to FDA guidance documents (U.S. Department of Health and Human Services, 2001). The method was linear from 0.1 μM (LLOQ) to 300 μM. Due to the low volume of plasma needed for quantitation, this method may be useful in applications where sample volume is a strong consideration, such as in measuring drug concentrations in neonates. The method was successfully applied to clinical plasma samples from adults in which metronidazole and 2-hydroxymetronidazole concentrations were quantified in plasma obtained from blood samples collected up to 48 hours post-dose.

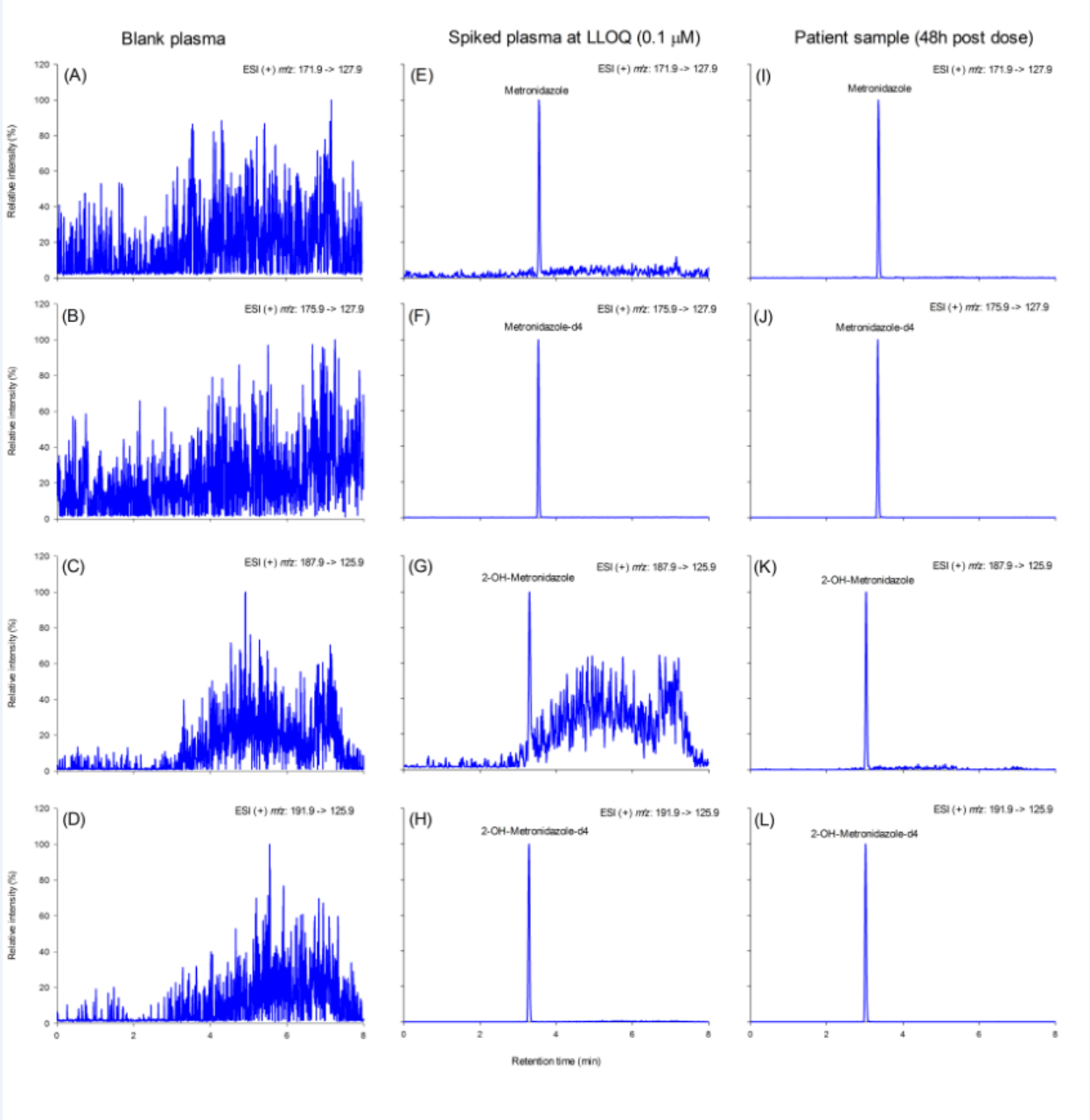

Figure 8. Representative chromatograms of blank human plasma (A-D), plasma spiked at LLOQ (0.1 μM) for metronidazole and 2-hydroxymetronidazole (E-H), and a representative plasma sample from a healthy adult volunteer 48 hours after receiving an oral dose of 500 mg metronidazole (I-L) demonstrating a metronidazole concentration of 0.8 μM and a 2-hydroxymetronidazole concentration of 0.6 μM. Each chromatogram is scaled to relative intensity of highest peak. Metronidazole, metronidazole-d_4, 2-hydroxymetronidazole (2-OH-metronidazole) and 2-hydroxymetroniadzole-d_4 (2-OH-metronidazole-d4) are listed in each column sequentially.

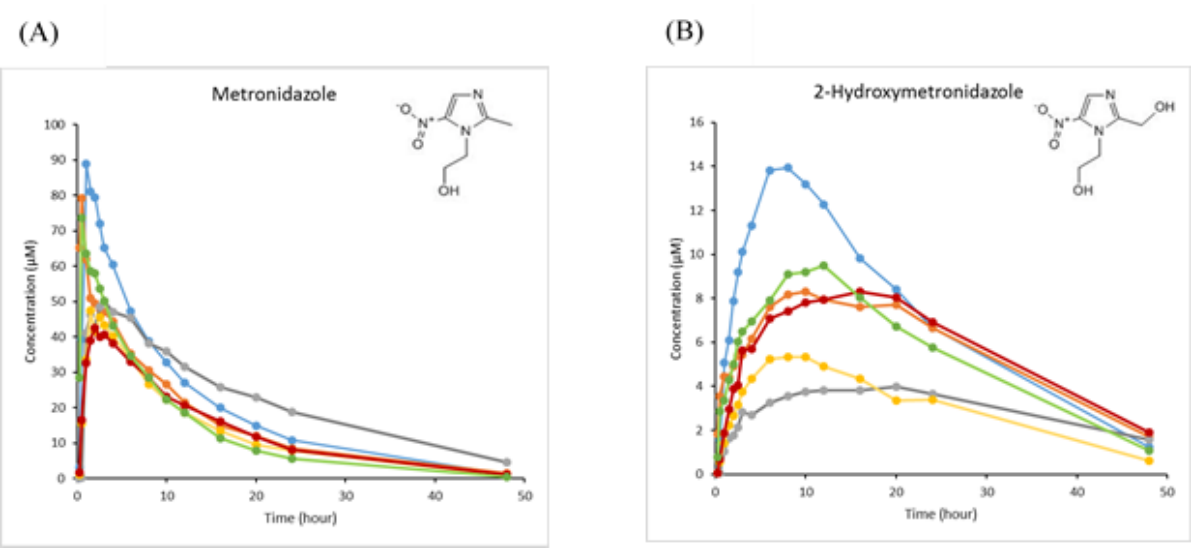

Figure 9. Concentrations of metronidazole (A) and 2-hydroxymetronidazole (B) in plasma samples obtained prior to dosing and at 0.25-48 hours post-dose from six healthy, adult volunteers given 500 mg oral metronidazole.

CHAPTER 4

VALIDATION OF METRONIDAZOLE AS A NOVEL, SAFE, CYP2A6 PHENOTYPING PROBE IN HUMANS

Based on: Stephani L Stancil, Robin E. Pearce, Rachel F. Tyndale, Gregory L. Kearns, J. Steven Leeder*, Susan Abdel-Rahman*. Establishing metronidazole as a novel, safe, CYP2A6 phenotyping probe in humans. *Manuscript in preparation*.
*Senior authorship is shared due to equal contribution by these two individuals

Introduction

CYP2A6 is a polymorphically expressed enzyme responsible for the biotransformation of nicotine and several therapeutic drugs (e.g., artemisinin, efavirenz, letrozole, tegafur). Inter-individual variability >100-fold has been reported for CYP2A6 activity *in vitro* and in *vivo* and has been shown to be influenced by genetic polymorphisms and exposure to both endogenous (e.g., estrogen) and exogenous compounds (e.g., clinical inhibitors isoniazid and methoxsalen). (Di et al., 2009).

Genetic variability of *CYP2A6* is quite prevalent. In line with the polymorphic patterns of other CYPs, certain racial groups are affected differentially (e.g., approximately 68% of East Asians carry at least one CYP2A6 variant). Variants conveying reduced activity are most predominant and are associated with reductions in activity (measured via nicotine clearance or metabolite ratio) of 25% (intermediate) to >50% (slow) compared with individuals who are homozygous wild type (Pan et al., 2015).

CYP2A6 variability has clinical consequences. It has typically been explored in the context of smoking behavior (e.g., cigarette consumption, topography-puff volume and frequency, and cessation success) and lung cancer risk. Specifically, those with alleles conferring reduced activity seem to require less frequent self-dosing of nicotine, which leads

to reduced exposure to procarcinogens found in tobacco, thus yielding a lower risk of lung cancer (Chenoweth et al., 2016; He & Feng, 2015; Moolchan et al., 2006; Tanner & Tyndale, 2017). In addition, CYP2A6 genetic polymorphisms are associated with variable exposure to several medications used globally to treat deadly diseases such as malaria, HIV and cancer (Tanner & Tyndale, 2017). CYP2A6 is responsible for the activation of the prodrug artesunate and contributes to the clearance of artemisinin. The consequences of variable CYP2A6 (e.g., decreased exposure vs. increased toxicity) differ based on the distinct roles that the enzyme plays in the metabolism of these antimalarial medications. Exposure to the antiretroviral efavrenz appears to be associated, at least in part, with CYP2A6 (McDonagh et al., 2015). *CYP2A6* reduced function alleles have been associated with decreased metabolism of letrozole both in patients with breast cancer and healthy post-menopausal women. Despite documented effects on substrate exposure, the impact of CYP2A6 variation on treatment outcomes for these medications is yet to be well described. In summary, *CYP2A6* is a pharmacogene with much left to be explored regarding the impact of genetic polymorphisms and variable activity on treatment outcomes.

Genotyping does provide some insight into enzyme function, yet substantial variability in activity is left to be accounted for even after *CYP2A6* genotype is known. Therefore, the phenotyping probe remains a valuable tool to guide future studies regarding risk assessment and therapeutic decisions related to variable exposure and response (Di et al., 2009).

Current *in vivo* phenotyping probes for CYP2A6 include nicotine, coumarin, and caffeine. Each of these carries significant limitations, either due to safety concerns, feasibility for use in non-smokers, or enzyme specificity. The current “gold standard” for

determining CYP2A6 phenotype is the measurement of the nicotine metabolite ratio (NMR) which detects 3-hydroxycotinine/cotinine (3HC/COT) in plasma, saliva or urine (D. Dempsey et al., 2004; Peamkrasatam et al., 2006). CYP2A6 accounts for approximately 80% of the c-oxidation of nicotine to form cotinine and 100% of the converstion of cotinine to 3-hydroxycotinine, thus 3HC/COT appears a more sensitive reflection of CYP2A6. 3HC/COT has proved convenient and reliable in smokers even with variable tobacco use (Hamilton et al., 2015; St Helen, Jacob, & Benowitz, 2013). Cotinine has a long half-life and 3-hydroxycotinine becomes formation dependent, thus the NMR can be measured at any time after cotinine has reached steady state in smokers. However, it is not a readily available measure in non-smokers and former smokers (Hamilton et al., 2015; St Helen et al., 2013). For these groups, researchers have administered deuterium-labelled cotinine directly to children (oral) or adults (intravenous) for determination of 3HC/COT; yet, this is not a compound readily available or easy to access (Benowitz et al., 2016; D. A. Dempsey et al., 2013; Zhu et al., 2013). Nicotine can be administered orally, buccally (i.e., gum), or intravenously to non-smokers followed by measurement of cotinine/nicotine (COT/NIC), cotinine/nicotine+cotinine (COT/NIC+COT) or 3HC/COT; however, problems still exist. COT/NIC and COT/NIC+COT are less specific for CYP2A6 due to the contribution from other enzymes such as aldehyde oxidase and CYP2B6 (Tanner & Tyndale, 2017). If using COT/NIC or COT/NIC+COT in smokers, administration of nicotine requires days of tobacco abstinence due to the long half-life of cotinine. The measurement of 3HC/COT after administration of nicotine in non-smokers would not yield comparable results with the NMR measured at cotinine steady state in smokers. Therefore, the biotransformation of nicotine to cotinine and then, further, to 3-hydroxycotinine (followed by measurement of COT/NIC or

3HC/COT) cannot be used as a CYP2A6 probe in a similar fashion in smokers and non-smokers.

Coumarin is a toxic anticancer agent no longer approved for use in the United States due to safety concerns. Its extremely rapid biotransformation also poses a technical challenge in sample acquisition.

Caffeine via the 8-hydroxylation of paraxanthine (17U/17X) has also been proposed as a probe for CYP2A6 activity; however, due to its cross-selectivity with CYP1A2, N-acetyltransferase 2 (NAT2) and xanthine oxidase (XO), poor CYP2A6 specificity results (Hakooz, 2009). Thus, the opportunity exists to find a novel CYP2A6 probe that would mitigate the risks and circumvent the limitations described above.

Criteria have been proposed to guide the selection and validation of phenotyping probes in humans. The candidate probe measure should provide a real-time reflection of complex factors affecting the enzymatic pathway of interest (e.g., inhibition or induction), and the probe drug itself should not directly impact the pathway in such a way that could mask the actual activity. Additional considerations when selecting a probe drug include specificity (for the reaction measured), safety, tolerability, cost, and commercial availability of the compound (Faber et al., 2005; Streetman et al., 2000).

Metronidazole (MTZ), an important antimicrobial agent with a unique spectrum of activity against both parasites and anaerobic bacteria (Samuelson, 1999), has properties that suggest it may serve as an excellent probe for CYP2A6 phenotype and thus, enzyme activity *in vivo*. Previously unknown until 2013, the main pathway of MTZ metabolism occurs through CYP2A6, which forms 2-hydroxymetronidazole (2HM). This reaction is highly specific, with CYP2A6 responsible for >96% of 2HM formation (Pearce et al., 2013). In

addition, MTZ is not predicted to lead to meaningful inhibition or induction of CYP2A6 based on *in vitro* evidence (presented in Chapter 2). MTZ is the drug of choice for treatment of *T. vaginalis*, the most common sexually transmitted disease globally, and thus it is widely available for clinical use (Bouchemal et al., 2017). MTZ is also a vital component of the treatment of *C. difficile*, amebic infections, and *H. pylori* and is used across the lifespan from neonates to elderly (Samuelson, 1999). It has been used therapeutically for over 50 years with significant evidence of favorable safety and tolerability profile, particularly at low oral doses. Finally, validation of MTZ as a pharmacologic probe to assess CYP2A6 activity would allow for mitigation of potential limitations associated with the use of currently available probes (e.g., nicotine, coumarin, and caffeine) and enable *in vivo* assessment of potential drug-drug interactions for CYP2A6 and its potential developmental dependence.

Therefore, the purpose of this prospective crossover study was to assess the validity of MTZ as a novel, safe CYP2A6 phenotyping probe (via measurement of 2HM/MTZ) against the current gold standard, 3HC/COT.

Results

Study Population

Characteristics of the study participants are listed in Table 10. Sixteen healthy adult volunteers completed all study procedures. The majority of participants (14/16) self-identified as white, non-Hispanic. One participant self-identified as Middle-Eastern and one as black. Four *CYP2A6* diplotypes were identified on genetic analysis and classified as wild type (*1/*1), intermediate (*1/*9) and slow (*1/*2; *1/*17) according to published guidance (Ho et al., 2009; McDonagh et al., 2012; Pan et al., 2015; Schoedel, Hoffmann, Rao, Sellers,

& Tyndale, 2004). Due to small numbers, the genotype-predicted intermediate and slow metabolizers were grouped as reduced metabolizers (RM) for analysis. Of note, one participant (*1/*9) was noted to have high levels of baseline cotinine in plasma (10x threshold for distinguishing smokers from non-smokers) and, after confirmation of tobacco use, was excluded from cotinine and 3-hydroxycotinine data analysis (Benowitz et al.,

Table 10

Demographics of Study Population

	Healthy participants (n=16)	
Variable	Value	Range
Gender (male/female)	9/7	-
Age (years)	30.6±13.9	18-56
BMI	24.9±2.4	19.7-28.4
Weight	76.5±12.2	50.1-93.3
CYP2A6 *1/*1, n (%)	10 (63%)	-
CYP2A6 *1/*2, n (%)	1 (6%)	-
CYP2A6 *1/*9, n (%)	3 (19%)	-
CYP2A6 *1/*17, n (%)	2 (13%)	-
Ethnicity, white, non-Hispanic, n (%)	14 (88%)	-

Note. BMI, body mass index; MTZ AE, metronidazole adverse event; Nic AE, nicotine adverse event

2009). All other participants had baseline (pre-dose) cotinine levels below the traditionally-excepted threshold for non-smokers indicating a negligible impact of cotinine on interpretation of this study data.

Pharmacokinetic Parameters of Metronidazole, Nicotine and Metabolites

Pharmacokinetic parameters of nicotine and cotinine in our study are within range of what is expected in healthy adults after dosing with nicotine gum (see Table 11) (Peamkrasatam et al., 2006). Of note, the study sampling scheme did not allow for determination of the pharmacokinetic parameters of 3HC due to the long half life of this metabolite. Expected genotype-phenotype relationships are seen with NIC, COT and 3HC. Figure 10 displays concentration-time curves for all entities evaluated, separated by genotype.

Pharmacokinetic parameters of MTZ and 2HM in our study are also within range of what is expected in healthy adults after similar oral doses (Gatchev et al., 2006; Jensen & Gugler, 1983; Jessa et al., 1997; Loft et al., 1986). Predicted genotype-phenotype relationships are present for MTZ and 2HM (see Figure 10). When RM were pooled together, 2-hydroxymetronidazole $AUC_{0\text{-}inf}$ was significant different compared with WT ($AUC_{0\text{-}inf}$ 262.6±44.9 μM*h for WT vs. 178.9±76.2 μM*h for RM, P<0.05). Additionally, significant differences were noted in 2-hydroxymetronidazole apparent Cmax (9.4±2.0 μM for WT vs. 5.4±2.5 μM for RM, P <0.05) between these two activity groups.

Given that 2HM formation is the most proximal phenotype for CYP2A6 using MTZ as a substrate, compartmental analysis was conducted to determine the rate-limiting step, formation or elimination, of 2HM. Compartmental analysis of the study population revealed the mean terminal half-life of 2HM of 15.0±5.6 h. When looking on the individual level, compared with WT, there was a trend towards longer terminal $t_{1/2}$ in RM although this did not reach statistical significance (WT 13.2±4.6 h vs. RM 18.2±6.1 h, P=0.12). Interestingly, there was a trend towards formation rate limited metabolite kinetics in RM individuals based

on parallel terminal phases of MTZ and 2-hydroxymetronidazole demonstrated in log-linear concentration-time curves along with plateaued 2HM/MTZ ratios after ~2 half-lives of parent drug elimination (i.e., 16 hours). This is in contrast to the characteristics of concentration-time curves observed in WT, which exhibited typical elimination rate limited kinetics.

Table 11

Pharmacokinetic Parameters of Parent Compounds and Metabolites in the Study Population

	Metronidazole N=16	2-Hydroxymetronidazole N=16	Nicotine N=16	Cotinine N=15
		Mean±SD (Range)		
Wt-adj dose (mg/kg)	6.7±1.2 (5.4-10.0)	-	0.027±0.005 (0.021-0.040)	-
Cl/F (L/h)	4.4±1.0 (2.7-6.2)	-	178.3±70.6 (34.5-296.6)	-
Cl/F/W (L/h/kg)	0.057±0.010 (0.039-0.072)		2.4±0.9 (0.5-4.2)	-
$AUC_{0\text{-}inf}$	701.6±172.5 (473.5-1091.4) µM*h	231.2±70.0 (108.1-340.8) µM*h	90.9±75.6 (41.6-357.7) nM*h	546.8±191.3* (131.6-820.0) nM*h
Tmax (hour)	1.2±0.8 (0.3-3.0)	9.9±3.7 (6.0-20.0)	0.8±0.3 (0.5-1.0)	4.1±1.8 (2.0-8.0)
Cmax	65.7±17.2 (42.0-91.6) µM	7.9±2.9 (3.3-13.9) µM	25.9±14.1 (11.8-70.6) nM	86.0±30.9 (19.8-132.4) nM
$T_{1/2}$ (hour)	8.5±1.8 (5.3-12.7)	12.6±2.6 (9.1-20.4)	2.3±0.7 (1.4-4.4)	15.2±7.2 (7.5-31.8)

Note. Data shown as mean±SD and (range). Wt-adj, weight-adjusted; Cl/F, apparent oral clearance; $AUC_{0\text{-}inf,}$ area under the curve; $T_{1/2}$, half-life; Pharmacokinetic parameters above determined by non-compartmental analysis
*Cotinine $AUC_{0\text{-}8,}$ area under the curve

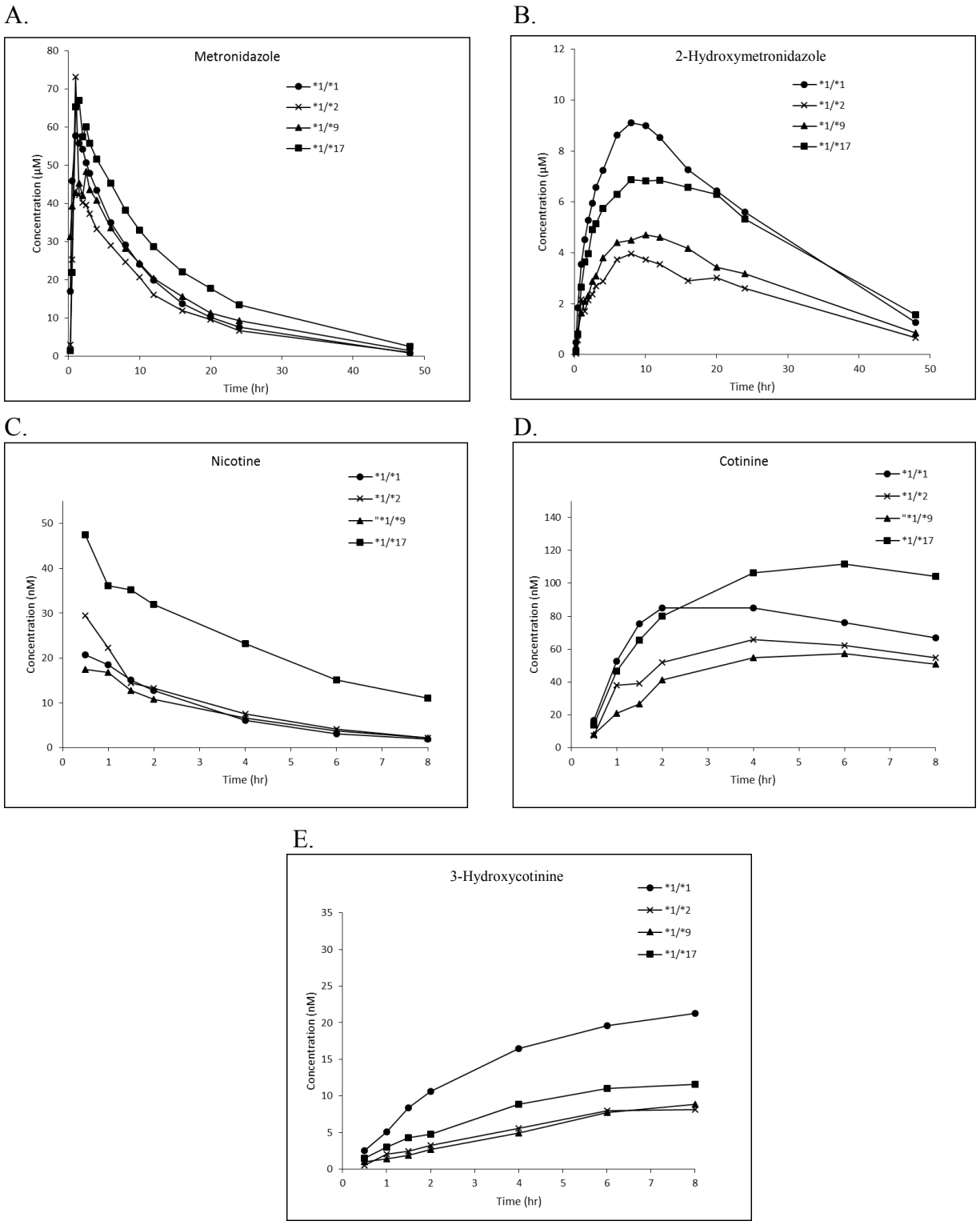

Figure 10. Concentration-time curves in plasma (mean±SD) in healthy volunteers with CYP2A6 WT (*1/*1, n=10) and CYP2A6 variants (*1/*2, n=1; *1/*9, n=3; *1/*17, n=2). A, B and C represent a total n=16. D. Cotinine and E. 3HC curves represent (total n=15, one individual with *1/*9 omitted due to confirmed tobacco use as described in the text).

Correlation of 2-Hydroxymetronidazole/metronidazole Ratio with Nicotine Probe Measures

Nicotine probe measures, COT/NIC and 3HC/COT, have both been used to understand CYP2A6 activity. When nicotine gum is administered to non-smokers, COT/NIC ratio in plasma has been measured 2 hours post dose (Nakajima et al., 2001; Peamkrasatam et al., 2006). In our cohort, COT/NIC measured at 2 hours post-dose correlated well with 2HM/MTZ between 2-20 hours (r=0.68-0.82; p<0.05). 3HC/COT is more widely used and studied than COT/NIC for insight into CYP2A6 activity. Dempsey et al suggests measuring 3HC/COT in the plasma of non-smokers between 2-8 hours after oral administration of labelled nicotine (Dempsey et al., 2004). In our study, the 2HM/MTZ ratio correlated well with 3HC/COT, measured after administration of nicotine gum, across a wide continuum of post-exposure time points (Table 12).

Although there is a wide range of potential time points of which to measure 2HM/MTZ based on the significant correlation with the nicotine probe measures, we wanted to look further into what time point may best capture formation of 2HM as we would expect this to be the optimal proxy for CYP2A6 activity. Because both formation and elimination rate limited kinetics are likely for 2HM in our study population, a 2HM/MTZ plasma ratio near the T_{max} of 2HM would likely provide optimal correlation with the 3HC/COT. The T_{max} is the time point reflecting equilibrium between formation and elimination and was 8.4±1.9 h for 2HM in our study population via compartmental analysis. Indeed, the 2HM/MTZ ratios at 8, 10 and 12 hours were found to correlate best 3HC/COT at 6 and 8 hours yielding the highest correlation coefficient of r=0.94 (p<0.0001). These 3HC/COT time points are within range of the "gold standard" after oral nicotine dosing suggested by Dempsey et al

(2004). Yet, numerous time points widely bracketing the T_{max} of 2HM demonstrated association (i.e., $r \geq 0.9$) in the matrix as demonstrated by medium gray shading. RM trended towards a later T_{max} compared with WT, but this did not reach significance (8.0±1.9 h in WT vs. 9.0±2.1 h in RM).

Table 12

Correlation Coefficients (r) of Metabolite/Parent Ratios (Probe Measure) in Plasma at Selected Time Points Post-Administration of Probe Drugs

		3HC/COT ratio in plasma (n=15)			
2HM/MTZ ratio in plasma (n=15)	**Hours**	**2**	**4**	**6**	**8**
	1	0.87	0.76	0.70	0.71
	1.5	0.88	0.78	0.74	0.75
	2	**0.92**	0.85	0.80	0.81
	2.5	0.91	0.89	0.86	0.86
	3	0.92	0.86	0.81	0.80
	4	0.91	0.86	0.83	0.83
	6	0.93	0.92	0.90	0.90
	8	0.89	0.93	0.94	0.94
	10	0.81	0.90	0.93	0.94
	12	0.77	0.87	0.93	0.94
	16	0.71	0.80	0.87	0.89
	20	<0.70	0.74	0.81	0.82
	24	<0.70	<0.70	0.75	0.76
	48	<0.70	<0.70	<0.70	0.73

When considering clinical utility (e.g., ease of use) the metronidazole and nicotine probe measures 2 hours post-dose correlated well with r=0.92, indicating a wide window of usage for 2HM/MTZ. Figure 11 further illustrates the correlation at this early time point by plotting individual ratios labeled with genotype-predicted phenotype.

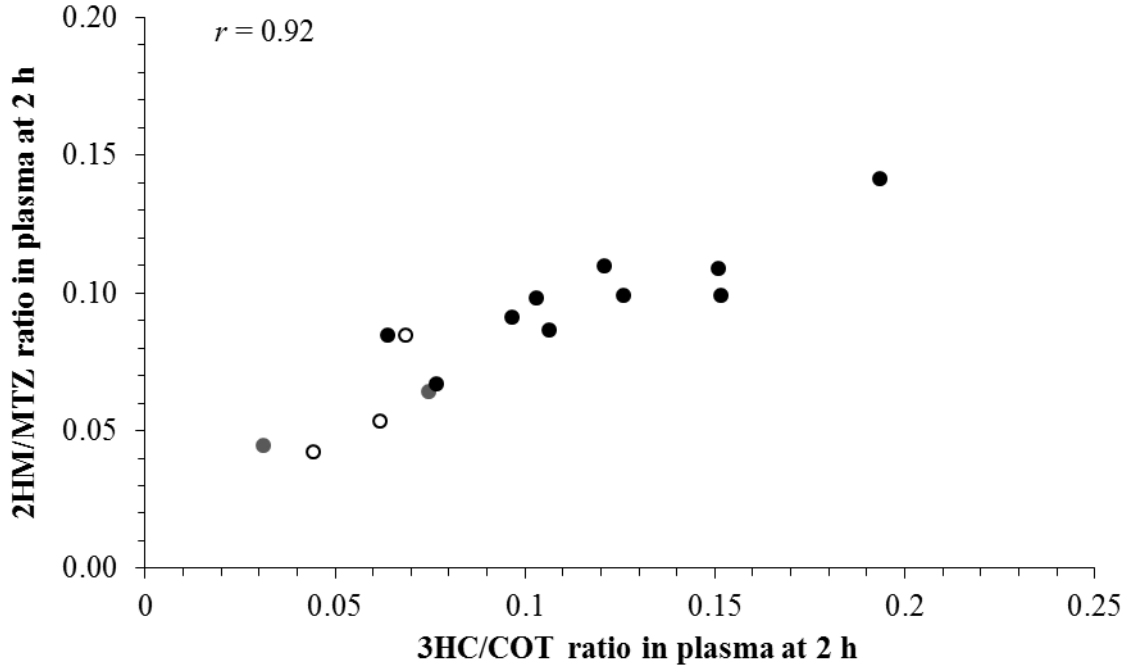

Figure 11. Correlation between metabolite/parent ratios (probe measures) of nicotine and metronidazole in healthy volunteers (n=15). Correlation coefficient (r) reflects comparison between 3HC/COT and 2HM/MTZ at 2 hours post dose (i.e., earliest 2HM/MTZ time point demonstrating $r \geq 0.9$ within 3HC/COT gold standard range). Solid circles represent CYP2A6 WT homozygotes. Gray circles represent *CYP2A6* genotype predicted intermediate metabolizers. Open circles represent *CYP2A6* genotype predicted slow metabolizers.

Performance of Genotype-predicted Phenotype

Nicotine probe measures, 3HC/COT and COT/NIC, have demonstrated separation between groups based on genotype prediction (Benowitz, Swan, Jacob, Lessov-Schlaggar, & Tyndale, 2006; Nakajima et al., 2001; Peamkrasatam et al., 2006). 2HM/MTZ mirrors this dichotomy in our study. Compared with WT (n=10), RM (n=5) demonstrated 2HM/MTZ ratios ≤58% and 3HC/COT ratios ≤56% at all time points examined in the study, with greatest separation observed in the 3HC/COT plasma ratio at 4 hr (43.6%, p=0.025) and in the 2HM/MTZ at 16 hr (50.7%, p=0.002). Table 13 lists the ratios for 3HC/COT and 2HM/MTZ by individual genotype along with the percent of activity compared with WT across the various time points. Figure 12 illustrates the genotype-predicted phenotype at 2

hours for both nicotine and metronidazole probe measures. Importantly, the two genotype groups (i.e., WT and RM) had significantly different 2HM/MTZ ratios measured at 2 hours, thus providing additional evidence for the clinical utility of this CYP2A6 probe measure early after MTZ administration.

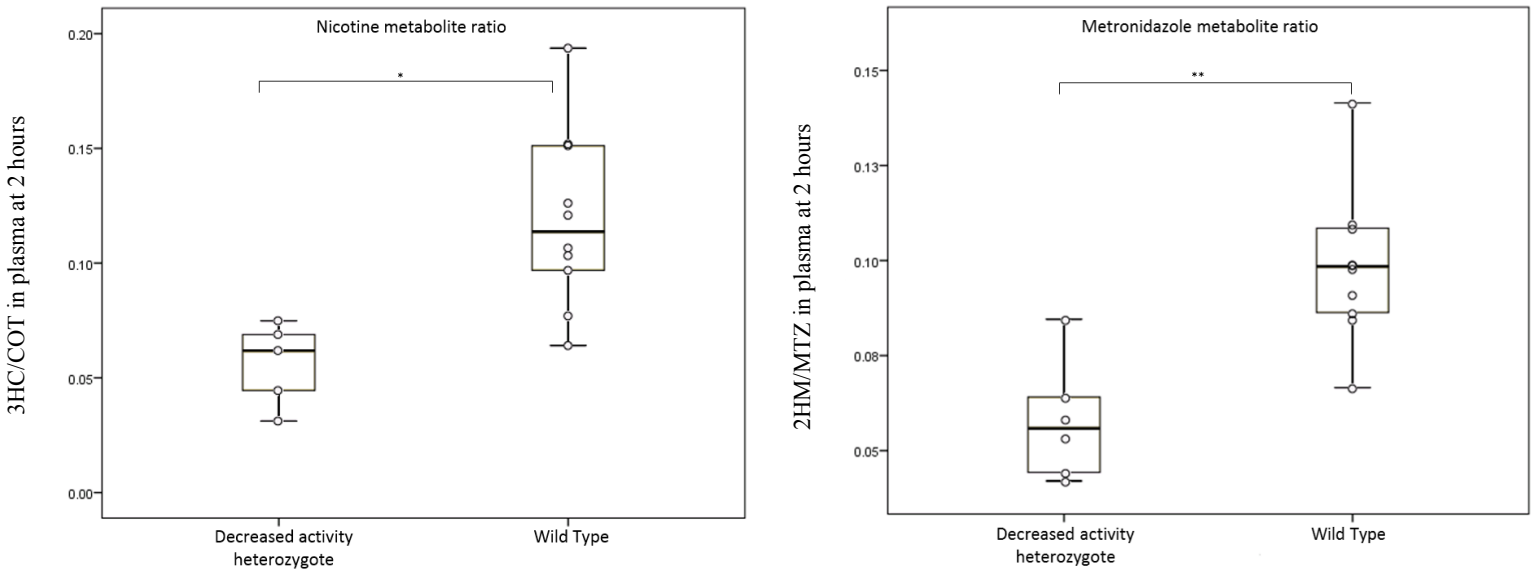

Figure 12. Difference between CYP2A6 wild type homozygotes (*1/*1) and CYP2A6 genotype predicted decreased activity heterozygotes (*1/*2, *1/*9, *1/*17) in metabolite/parent ratio in plasma at 2 hours. Nicotine metabolite ratio (n=15). Metronidazole metabolite ratio (n=16). *p<0.05, **p<0.01

Table 13

Probe Measures at Various Time Points by Individual Genotype

	Hr	*1/*1 (mean, n=10)	SD	*1/*2 (n=1)	*1/*9 (mean, n=2)	SD	Activity of WT (%)	*p* value (*1/*9 vs. *1/*1)	*1/*17 (mean, n=2)	SD	Activity of WT (%)	*p* value (*1/*17 vs. *1/*1)
COT/ NIC	**2**	6.85	2.40	3.91	3.21	2.46	47	0.079	2.74	1.69	40	**0.046**
	4	15.67	7.26	8.74	6.75	4.90	43	0.134	5.97	4.52	38	0.105
	6	27.61	10.57	15.09	13.52	10.95	49	0.117	10.60	8.49	38	0.060
	8	47.43	30.87	25.38	23.06	22.42	49	0.321	14.95	12.94	32	0.186
3HC/ COT	**2**	0.119	0.039	0.062	0.053	0.031	45	**0.048**	0.06	0.02	48	0.055
	4	0.184	0.058	0.085	0.064	0.054	35	**0.023**	0.08	0.04	43	**0.037**
	6	0.246	0.076	0.128	0.102	0.073	41	**0.033**	0.10	0.06	39	**0.027**
	8	0.310	0.098	0.149	0.129	0.101	41	**0.038**	0.11	0.08	35	**0.023**
2HM/ MTZ	**2**	0.098	0.020	0.053	0.054	0.014	55	**0.014**	0.06	0.03	64	0.056
	2.5	0.118	0.024	0.060	0.062	0.010	52	**0.010**	0.08	0.05	64	0.066
	3	0.139	0.032	0.072	0.073	0.019	52	**0.022**	0.09	0.05	64	0.087
	4	0.169	0.040	0.087	0.090	0.025	53	**0.026**	0.11	0.07	63	0.099
	6	0.252	0.065	0.129	0.130	0.035	52	**0.031**	0.14	0.10	55	0.059
	8	0.318	0.075	0.160	0.158	0.060	50	**0.019**	0.18	0.12	56	0.052
	10	0.387	0.098	0.180	0.188	0.073	49	**0.023**	0.22	0.16	56	0.062
	12	0.444	0.108	0.221	0.211	0.074	47	**0.016**	0.25	0.18	56	0.060
	16	0.560	0.151	0.242	0.250	0.105	45	**0.021**	0.33	0.26	59	0.098
	20	0.660	0.171	0.312	0.276	0.109	42	**0.013**	0.42	0.35	64	0.145
	24	0.777	0.213	0.382	0.311	0.159	40	**0.016**	0.53	0.47	68	0.230

Note. Light gray shading represents columns with ratio values. Dark gray shading represents columns with % activity compared with WT (*1/*1) mean ratios.

Variables Associated with CYP2A6 Probe Measures and Metronidazole Exposure

The relative contribution of CYP2A6 genotype-predicted phenotype and non-genetic factors to the observed variability in phenotypic measures of CYP2A6 activity, 3HC/COT and 2HM/MTZ, was assessed by multiple linear regression including *CYP2A6* genotype, gender, BMI and age as explanatory variables independently in a univariate analysis as well as all factors incorporated into a full multivariate model (Table 14). For 3HC/COT and 2HM/MTZ, *CYP2A6* genotype was the single most important factor contributing to variability in the metabolite ratios, accounting for 52% (p=0.015) and 55% (p=0.007) of the observed variability, respectively. None of the demographic variables contributed significantly to the model. In contrast, *CYP2A6* genotype accounted for 36% and only 8% of the observed variability in 2-OH-MTZ $AUC_{0\text{-inf}}$ and MTZ $AUC_{0\text{-inf}}$, respectively, further supporting use of the metabolite ratio as the preferred phenotype when MTZ is used to assess CYP2A6 phenotype.

Adverse Events

Adverse events considered to be related to the study drug occurred in six participants who complained of dizziness after nicotine administration (38%). These events were mild and self-limiting, with no significant intervention required. No participants experienced any adverse events post metronidazole administration. RM were more likely to experience nausea or headache post-nicotine administration than WT (RR 3.3, 95% CI 0.85-13.02, $p<0.05$).

Table 14

Variables Associated with CYP2A6 Probe Measures and MTZ Exposure

Predictor	r^2	B Coefficient	*p* value	Full Model B Coefficient (95% CI)	Full model *p* value
		3HC/COT at 6 h (n=15)			
CYP2A6 genotype	0.52	0.141 (0.060-0.223)	**0.002**	0.135 (0.032-0.238)	**0.015**
Gender	0.36	-0.110 (-0.199-(-)0.021)	**0.019**	-0.018 (-0.124-0.088)	0.712
BMI	0.18	-0.016 (-0.037-0.005)	0.120	-0.001 (-0.018-0.017)	0.922
Age	0.16	0.003 (-0.001-0.006)	0.136	0.003 (-0.001-0.006)	0.147
All factors	0.71				**0.009**
		2HM/MTZ at 8 h (n=16)			
CYP2A6 genotype	0.55	0.152 (0.074-0.230)	**0.001**	0.148 (0.051-0.246)	**0.007**
Gender	0.34	-0.116 (-0.209-(-)0.022)	**0.019**	-0.005 (-0.114-0.105)	0.924
BMI	0.13	-0.015 (-0.038-0.007)	0.167	0.000 (-0.017-0.018)	0.989
Age	0.18	0.003 (-0.001-0.007)	0.104	0.003 (-0.001-0.007)	0.114
All factors	0.72				**0.005**
		MTZ $AUC_{0\text{-}inf}$ (n=16)			
CYP2A6 genotype	0.08	-98.127 (-287.687-91.432)	0.286	-260.021 (-500.64-(-)19.403)	**0.037**
Gender	0.01	-26.252 (-218.628-166.124)	0.774	-251.512 (-521.53-18.501)	0.065
BMI	0.01	-4.797 (-45.660-36.065)	0.805	-23.992 (-67.028-19.044)	0.245
Age	0.03	-1.981 (-9.027-5.066)	0.556	-7.685 (-16.704-1.333)	0.087
All factors	0.39				0.202
		2HM $AUC_{0\text{-}inf}$ (n=16)			
CYP2A6 genotype	0.36	83.687 (19.368-148.006)	**0.014**	5.487 (-38.124-49.099)	0.787
Gender	0.38	-84.444 (-145.995-(-)22.893)	**0.011**	-66.621 (-115.56-(-)17.682)	**0.012**
BMI	0.62	-22.749 (-33.052-(-)12.445)	**<0.001**	-20.181 (-27.981-(-)12.381	**<0.001**
Age	0.11	1.677 (-1.055-4.409)	0.209	0.121 (-1.514-1.755)	0.874
All factors	0.88				**<0.001**

Note. Rationale for inclusion of specific time points in analysis above: 2HM/MTZ at 8 hours was chosen as it was the earliest time point with the highest correlation *(r*=0.94) with 3HC/COT (which occurred earliest at 6 hours).

Discussion

As previously mentioned, validation criteria for a phenotyping probe include accurate reflection of the enzyme activity, specificity of the reaction measured, and safety, tolerability, and accessibility of the drug. 2HM/MTZ appears to provide a good reflection of CYP2A6 activity given its excellent correlation with 3HC/COT across numerous time points. Even at 2 hours post-dose, 2HM/MTZ correlated extremely well ($r>0.9$) with 3HC/COT. 2HM/MTZ yielded a higher correlation with 3HC/COT, compared with COT/NIC, providing additional evidence for similar specificity of MTZ conversion to 2HM by CYP2A6 *in vivo*. This is supported by *in vitro* evidence that demonstrated the affinity and specificity of metronidazole for CYP2A6 (Pearce et al., 2013).

In our study, significant differences were seen between dichotomous genotype-predicted phenotypes (i.e., WT and RM) measured by 2HM/MTZ ratios in plasma at 2-24 hours ($P<0.01$). Interestingly, *CYP2A6* genotype alone accounted for more than half of the variability of 2HM/MTZ and 3HC/COT (see Table 14). When gender, BMI and age were added to the model, 71% and 72% of the variability in 3HC/COT and 2HM/MTZ, respectively, could be explained, but none of these factors was significant in the full model. 2HM/MTZ and 3HC/COT appear to have a similar dependence on CYP2A6 in the multivariate analysis which provides additional evidence for the specificity of 2HM/MTZ being comparable to 3HC/COT as measure of CYP2A6 activity. Clearly, additional factors leading to variability in these probe measures and, by extension CYP2A6, must be identified. Possibilities include dietary components not yet known to affect CYP2A6,

endogenous variables leading to altered CYP2A6 expression and/or function, or novel CYP2A6 genetic variants not yet characterized.

Formation rate limitation of the metabolite is ideal when measuring the product of a reaction catalyzed by an enzyme. Characteristics of metabolite kinetics can be difficult to ascertain on an individual level. It is logical to assume a trend towards formation rate limitation of the specific reaction when the enzyme is only partially functional or less abundant as would be the case with genetic variants conferring reduced activity. If formation rate limitation was present, we would expect decreased exposure of the metabolite along with parallel terminal phases of the parent and metabolite. This is in fact what we saw for 2HM in most individuals with reduced activity variants. If elimination rate limitation was present, we would expect increasing metabolite/parent ratios over time rather than a plateauing of ratios after 2-3 half-lives (Houston, 1981). That was demonstrated for 2ohm/MTZ in all WT individuals. Thus, formation of 2ohm appears sensitive to changes in CYP2A6 function, adding another prong of evidence for the use of 2HM/MTZ as a specific measure of variable activity.

CYP2A6 variability has also been of interest in prior work related to smoking behavior, cessation success, and lung cancer risk (Park et al., 2017; Patel et al., 2016; Tanner & Tyndale, 2017). In active smokers, 3HC/COT is a convenient measure of CYP2A6 activity as it can be measured independent of time once cotinine is at steady-state and, thus, can allow for investigation into associated outcomes. However, in non-smokers such as school age children and infants as well as former smokers, a non-nicotine method has advantages such as avoiding toxicity, addictive potential, and issues related to legality of nicotine. To obviate the negative aspects of nicotine, investigators have used cotinine

administered directly to humans despite its incomplete safety profile in both children and adults and its relative inaccessibility in a therapeutic context (D. A. Dempsey et al., 2013). In contrast, metronidazole at low doses such as that used in this study has a large body of evidence to document safety across the lifespan including lack of teratogenicity to developing human fetuses if taken during pregnancy. In addition, 2HM/MTZ ratio values could be directly compared in smokers and non-smokers. (Lamp, Freeman, Klutman, & Lacy, 1999; Sheehy, Santos, Ferreira, & Berard, 2015). Hence, the 2HM/MTZ ratio has the potential to shed light on the impact of ontogeny on CYP2A6 activity regardless of smoking status and also, as a potential "tool" to understand the risks for smoking initiation and the effects of environmental nicotine exposure (e.g., active vs. passive smoking, vaping in adolescents and young adults).

The study design was chosen based on published work with nicotine, the *in vitro* (McDonagh et al., 2012) and *in vivo* assessment of CYP2A6 activity (Wang et al., 2017), and clinical feasibility pertaining to the investigation of metronidazole as a phenotyping probe. Although nicotine pharmacokinetics were well-characterized (Benowitz et al., 2009), the long $t_{1/2}$ of cotinine (16 -20h) (Benowitz et al., 2006; D. Dempsey, 2004; D. A. Dempsey et al., 2013) and 3-hydroxycotine (6.6h) (Benowitz & Jacob, 2001) make full pharmacokinetic analysis of these metabolites challenging in a clinical context. In contrast, the pharmacokinetic properties of metronidazole and its CYP2A6 catalyzed metabolite in addition to other properties of the drug (e.g., use/safety profile across development, accessibility, ease of administration) made it an attractive candidate for investigation as a probe. It is important to note that absolute values for the 3HC/COT ratio in plasma reported in our study following administration of nicotine gum in non-smokers are not directly

comparable to the nicotine metabolite ratio (3HC/COT) measured at cotinine steady state in smokers. Yet, the 3HC/COT measured between 2-8 hours in non-smokers after oral nicotine is highly specific for CYP2A6 and has been previously reported to correlate well with nicotine clearance. Thus, it was used as the main comparator. Finally, the size of our study cohort did not permit a genotype-directed approach to study. It did enable us, however, to explore the genotype-phenotype relationship for CYP2A6 activity. Future genotype directed studies could further characterize this potential aspect of metronidazole but was beyond the scope of this present study.

In conclusion, metronidazole appears to be a well-tolerated probe drug, and the 2HM/MTZ ratio is a specific and accurate biomarker of CYP2A6 activity in healthy adults. The benefits of using this probe to further describe CYP2A6 variability include ease of use (e.g., 2 hours post-dose), drug availability, a well-defined, excellent safety profile, and potential for use in all ages.

Methods

Subjects

Healthy, non-pregnant adults (n=16) between the ages of 18-65 years were eligible for enrollment. Participants were considered "healthy" if they had a BMI between 18.5-29.9; no clinically significant abnormality on screening laboratory evaluation (complete blood count, basic metabolic profile, hepatic function test and urine pregnancy test-if applicable) or physical exam; no known pathologic condition that could affect absorption, distribution, metabolism, or excretion of study drug (e.g., liver or kidney disease); and no previous gastrointestinal surgery that could influence function of the gastrointestinal tract (e.g., bariatric procedure). Participants were required to be tobacco free (at least 30 days of

abstinence), using no over-the-counter medications for 48 hours prior to or during the study, and taking no herbal supplements or prescription medication (except for hormonal contraceptive agents) for at least 14 days prior to and during the study. Participants were also asked to refrain from consumption of foods or beverages containing alcohol, caffeine, or grapefruit juice within 48 hours prior to or during the study. Participants were recruited through blast email notifications to hospital employees, postings on social media, bulletins at nearby universities, and notification on an active study registry maintained by the CTSA at the University of Kansas Medical Center. The study, including recruitment strategy and materials, was approved by the Institutional Review Boards at Children's Mercy and the University of Missouri-Kansas City.

Study Design

We conducted an open-label, randomized, two period cross-over study to evaluate the pharmacokinetic profiles of metronidazole and correlate the metabolite/parent ratio with that of nicotine to assess validity of metronidazole as a CYP2A6 phenotyping probe. This study was conducted with a minimum two-week washout interval separating each treatment period to minimize the degree of inter-individual variability that would be observed with an independent cohort design. Randomization was utilized to determine administration sequence of study drugs.

Before dosing, participants underwent full physical examination, vital signs, and laboratory evaluation (as above) interpreted by a licensed health care provider. Blood was also obtained for *CYP2A6* genotyping. Participants arrived at the clinical research unit in the morning on the day of study after an overnight fast. During the nicotine study arm, participants remained in the clinical research unit 8 hours post-dose. Nicotine gum (2 mg)

was chewed for 30 minutes, with instructions to chew for 20 out of every 30 seconds which was observed by study staff. Plasma samples for the quantitation of nicotine, cotinine, and trans-3-hydroxycotinine were obtained pre-dose and 0.5, 1, 1.5, 2, 4, 6, and 8 hours after nicotine administration. During the metronidazole study arm, participants spent the first 24 hours in the clinical research unit and then returned 48 hours post-dose for final sample collection. Metronidazole tablets (500 mg) were administered by mouth. Participants were allowed water *ad libitum* throughout the study with the exception of the immediate two-hour post nicotine gum administration. Standard hospital meals were provided starting 2 hours after drug administration while in the clinical research unit and were absent caffeine and grapefruit juice. Plasma samples for the quantitation of metronidazole and 2-hydroxymetronidazole were obtained pre-dose and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, 20, 24, and 48 hours after metronidazole administration. Participants were restricted from strenuous physical activity during the blood sampling period.

Bioanalytical Method

Concentrations of all analytes in plasma were determined by UPLC-MS/MS (Waters Acquity TQD, Milford, MA). The analytical method for determination of metronidazole and 2-hydroxymetronidazole was developed and validated according to the FDA guidelines for a bioanalytical assay (Stancil et al., n.d.). Briefly, samples were prepared for injection onto UPLC-MS/MS using solid phase extraction in a 96-well plate (Oasis Hydrophilic-Lipophilic Balance microelution plate; Waters Corp., Milford, MA). Analytes were separated on a Waters Acquity BEH reversed phase column (BEH 1.7 um, 2.1 x 50 mm) using gradient elution and mobile phases consisting of 0.1% formic acid-methanol (A) and 0.1% formic acid-water (B) with total run time of 8 minutes. Deuterium-labeled compounds served as

internal standards. LLOQs for metronidazole and 2-hydroxymetronidazole were both 0.1 μM.

The analytical method for determination of nicotine, cotinine, and trans-3-hydroxycotinine in plasma was adapted from a previously published method. The modified method used a lower plasma volume (100 μL vs. 500 μL) with proportional reduction in wash volumes from 1000 to 200 μL (Dobrinas et al., 2011). After elution, samples were evaporated to dryness and then reconstituted in 100 μL 100% acetonitrile. Deuterium labeled analytes served as internal standards. Inter-day variability was within acceptable limits according to the FDA guidelines for a bioanalytical assay (CV<15% and accuracy 85-115%; lower limit of quantification- LLOQ CV<20% and accuracy 80-120%). LLOQs for nicotine, cotinine and trans-3-hydroxycotinine were 1, 5, 1 nm, respectively.

Pharmacokinetic Analysis

Pharmacokinetic analyses were conducted using Kinetica version 5.0 (Thermo Scientific, Philadelphia, PA). Pharmacokinetic parameters for each participant were estimated based on non-compartmental analyses. The area under the plasma concentration vs. time curve during the sampling period ($AUC_{0\text{-}t}$) was calculated using the mixed log-linear rule and extrapolated (C_{last}/λ_z) to infinity ($AUC_{0\text{-}inf}$). Weight-adjusted apparent oral clearance was calculated by dividing the apparent oral clearance (CL/F) by weight (kg). Rate constants for 2HM were determined by compartmental analysis to fully characterize formation and elimination of this metabolite. Half-lives were calculated using the following equations: $t_{1/2\ elim} = 0.693/\lambda_z$ and $t_{1/2\ form} = 0.693/ka$. The apparent terminal elimination rate constant (λ_z) and formation rate constant (ka) were determined from weighted linear least squares regression of log-linear plasma concentration versus time curves. Each curve was

assessed for goodness of fit based on mathematical criteria (objective function, Akaike, Schwartz). Coefficients of variation (%CV <30%) was used to determine final parameter estimates from the model-dependent analyses.

CYP2A6 Genotyping

Genomic DNA was extracted from whole blood (3 mL) using Qiagen All-Prep DNA/RNA mini kit (Valencia, CA) according to manufacturer's recommendations. Isolated genomic DNA samples were frozen at -80˚C and shipped to the University of Toronto for *CYP2A6* genomic inquiry of known allelic variants (*2, 4, 7, 9, 12, 17, 20, 23-28, 31, 35) using TaqMan assays. Because no participants were found to carry a *7 allelic variant, samples were not tested for *8 and *10 (Wassenaar et al., 2015; Wassenaar, Zhou, & Tyndale, 2016).

Safety

Occurrence of any adverse events, subsequent actions taken, and outcomes observed were assessed throughout all study days. Determination of relatedness to the study drug was done by the principal investigator.

Statistical Analysis

Power analysis revealed that 13 subjects would be required to detect a difference in correlation coefficient (r) of 0.9 compared with 0.5 at 80% power. Therefore, we enrolled 16 subjects to exceed minimum power analysis calculations while allowing for attrition. Correlation analyses were utilized to test for agreement between the two measures (nicotine and metronidazole). A null value of 0.5 was chosen as a threshold for agreement, meaning a correlation coefficient less than 0.5 would lead us to conclude there is insufficient agreement between these measures to validate metronidazole. Power analysis was based on a true

correlation coefficient of 0.9; this value implies that one measure can explain 81% of the variability in the specific measure.

Student's *t* (paired, two-tailed) and Chi-square tests were utilized to evaluate differences between groups using Excel 2013 (Microsoft, Redmond, WA) and OpenEpi (Dean, Sullivan, & Soe, 2010), respectively. ANOVA and multiple linear regression were performed using SPSS version 23 (IBM Corp., Armonk, NY) to describe the relationships between variables of interest. The level of significance accepted for all statistical analyses was $\alpha = 0.05$.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

CYP2A6 is a polymorphically expressed enzyme. Variation in this pathway has been associated with smoking behavior, cessation success, and lung cancer risk. In addition, differential drug exposure in individuals with decreased activity alleles has been demonstrated for numerous medicatons, including the anti-infectious and chemotherapeutic agents, efavirenz, artesunate, letrozole, and others. The effect of CYP2A6 variability on treatment outcomes is yet to be fully elucidated and requires ongoing study. Thus, a tool such as a phenotyping probe that can describe CYP2A6 variability in humans is important to gain insight into health risks and the potential need for individually tailored therapeutics. Current CYP2A6 probes are limited due to safety, accessibility, general applicability, and/or enzyme specificity. Therefore, the enclosed studies sought to validate a novel biomarker to understand variability in CYP2A6 activity.

The overall purpose of this research was to evaluate the use of metronidazole as a CYP2A6 phenotyping probe in humans and required a step-wise approach as summarized in the subsequent paragraphs.

To establish the validity of this novel probe, metronidazole was compared with the "gold standard," nicotine. When deciding the potential utility of a new phenotyping probe, the ability of the drug to inhibit or induce the pathway of interest must be explored. Ideally, the drug should not cause any relevant interference with the enzyme and be able to reflect any variation in activity present in the system. To accomplish this goal, the effect of metronidazole on *CYP2A6* gene expression and activity was investigated *in vitro*. The effect of nicotine was also explored, as there was limited evidence available regarding its behavior

in vitro. Metronidazole and nicotine had minimal effects on CYP2A6 expression or activity *in vitro* at therapeutically relevant concentrations. Therefore, the effects mediated by metronidazole and nicotine on CYP2A6 activity would be expected to have limited clinical consequences.

An analytical method was developed to simultaneously quantitate metronidazole and its primary metabolite, 2-hydroxymetronidazole in human plasma. This method required a low volume of plasma (10 μL) and achieved lower limits of quantitation (LLOQ) two to three times more sensitive than previously described. This novel method also has the potential to be high-throughput if paired with automated technology.

Finally, metronidazole was compared to nicotine for validation of its use as a phenotyping probe in humans. Metronidazole used as a probe drug was well-tolerated with no adverse events reported. Performance of genotype-predicted phenotypes were also explored to extend the understanding of substrate specific effects on this relationship. The metronidazole metabolite ratio (MMR) proved highly specific for CYP2A6 and robust with a wide window of use. This novel probe measure was also able to dichotomize individuals with homozygous wild type from decreased activity heterozygotes in a way that mirrored the nicotine metabolite ratio (NMR). Taken together, this study provided evidence for the use of the MMR as a safe, specific and readily available biomarker of CYP2A6 activity in humans with the potential for use across the lifespan and importantly, regardless of smoking status.

Future studies evaluating the use of metronidazole as a probe of CYP2A6 activity in special populations would be useful to further investigate the performance of this tool. Neonates receiving metronidazole therapeutically are one such clinical cohort where additional understanding of variability in exposure may provide important information when

making treatment decisions. Additionally, should new chemical entities with narrow therapeutic indices prove to be CYP2A6 substrates, the use of metronidazole administered first as a phenotyping probe may provide insight to the scientist and/or clinician to guide implementation of precision therapeutics. Using knowledge gained from studies in adult and adolescent smokers regarding CYP2A6 activity and correlates of smoking behavior and cessation success, metronidazole phenotyping may provide insight *a priori* regarding risk for smoking behavior (e.g., age of initiation, susceptibility to carcinogenic compounds in tobacco). Knowledge regarding risk could be paired with behavioral techniques to determine if such a multifaceted approach would be effective in delaying age of smoking onset and/or preventing tobacco use in at-risk young people.

In summary, the work described herein establishes metronidazole as a novel probe drug and the use of 2HM/MTZ as a biomarker of CYP2A6 variability in humans, thus providing a tool to understand human diversity and potentially, improve health outcomes in a diverse pool of individuals.

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VITA

Stephani L. Almai Stancil was born March 22, 1985 in Kansas City, Missouri to parents Janette Kay (Jan) and Manuchihr Almai. She was valedictorian of her graduating class in 2002 at Heartland High School in Belton, Missouri. She completed her undergraduate education in 2006 majoring in nursing at the University of Kansas (KU) and graduated with highest distinction (top 3% of her class). She worked as a staff nurse in the Pediatric Intensive Care Unit at Children's Mercy Kansas City before proceeding to graduate school. She received her degree as a Family Nurse Practitioner from KU in 2009 and worked as a teaching assistant throughout her time as a graduate student. She was awarded the Graduate Student Clinical Excellence Award for her performance during clinical rotations. After graduation, she obtained national board certification as a Family Nurse Practitioner, which she continues to hold, and has clinical licensure to practice in both Kansas and Missouri. She continues to work as a nurse practitioner in the Division of Adolescent Medicine at Children's Mercy Kansas City.

Ms. Stancil began her Ph.D. in pharmacology and pharmaceutical science at the University of Missouri-Kansas City (UMKC) in the Fall of 2011. While pursuing her Ph.D., she has presented numerous abstracts at national scientific meetings including the Society of Adolescent Health and Medicine (SAHM), Pediatric Academic Societies (PAS), the American Society of Clinical Pharmacology and Therapeutics (ASCPT) and the American Society for Pharmacology and Experimental Therapeutics (ASPET). She published original research in the peer-reviewed journal, *Pediatrics.* She is a member of SAHM and ASCPT.

Upon completion of her degree requirements, Ms. Stancil plans to continue pursuing her research interests that broadly include striving to understand variability in drug response

in adolescents leading to adverse reactions or treatment failure, thus providing answers (and more questions) to help further the implementation of precision medicine.