Determination of Absolute Protein Content of Hepatic CYP4F Enzymes in Human Liver Microsomes Using LC/MS/MS Methodology and Comparison with Immunoquantification and Enzyme Activity

By

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Abstract

The purpose of the work presented herein was to determine the absolute protein content of multiple cytochrome P450 (CYP) enzymes in individual donor human liver microsome (HLM) samples. The CYP4F subfamily of enzymes have recently been identified to be involved in the metabolism of endogenous compounds (arachidonic acid, leukotriene B_4), nutrients (vitamin K1 and vitamin E), and xenobiotics (parfuramidine (DB289), DB1230, fingolimod). The CYP3A subfamily of enzymes are known to metabolize a wide variety of physiologically and pharmaceutically important substances. The determination of the absolute enzyme protein content and the inter-individual variability in the expression of these enzymes is important in understanding the effects they may have on the disposition of both endogenous and exogenous substances. Therefore, the absolute enzyme protein content in 20 individual donor HLM was determined by LC/MS/MS for CYP4F2, CYP4F3B, CYP3A, CYP3A4, and CYP3A5. The contribution of CYP4F enzymes to the overall hepatic CYP "pie" was also determined. The observed enzyme protein values were then correlated with immunoquantified protein content and enzyme activity. The enzyme protein contents determined by LC/MS/MS were well correlated with immunoquantification results ($r^2 \geq 0.60$) for both CYP4F and CYP3A. The CYP4F enzymes displayed significantly less (~2- to 4-fold) variability than did the CYP3A enzymes (~7- to 20-fold). The CYP4F protein contents did not correlate with primary metabolite formation rates for DB289 or DB1230. Chemical inhibition experiments were performed which provided additional evidence for the metabolism for DB289 and DB1230 by enzymes other than CYP4F enzymes. However, a lack of improved correlation for the chemical inhibition experiments suggest that the poor correlation observed for CYP4F protein content and metabolic activity is not solely due to the contributions of additional enzymes. The LC/MS/MS

methodology used for the current study has thus been shown to provide a rapid and reproducible (CV $\leq$ 24%) method for quantitation of enzyme protein expression level that obviates the need for specific antibodies for immunoquantification, a major problem for the less commonly studied enzymes that share significant sequence homology such as the CYP4F enzymes.

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Chapter One: Research Objectives and Chapter Summaries

1.1 Research Objectives

Since the discovery of the CYP superfamily of enzymes approximately 30 years ago, there has been a continued interest in characterizing the distribution and expression of individual CYP enzymes in a wide variety of tissues for preclinical species and humans. By the early 1990's, the major CYPs involved in drug metabolism had been identified and the scientific community was beginning to understand that the expression levels of these enzymes showed a significant degree of inter-individual variability. This high degree of variability reduced the accuracy with which *in vitro* assays and preclinical animal models could be used to predict pharmacokinetic parameters (i.e., clearance, AUC, bioavailability) in humans. Additionally, it was observed that a given enzyme profile may change significantly within an individual as a result of many pathological conditions, such as cancer, or upon exposure to various environmental factors that can induce or inhibit enzyme expression and/or activity. This variability can have significant effects on the success of drug therapies and as such, characterization of changes in CYP levels within a population or individual would be of great value.

Critical to the characterization of enzyme variability is the ability to accurately and specifically quantitate a particular protein from a complex biological mixture. Historically, this has been achieved by analysis of mRNA levels (*real time* RT-PCR) or through the use of antibody-based methods. These techniques are limited by low throughput, cross-reactivity of antibodies with highly homologous proteins, and the fact that mRNA expression does not always correlate well with protein expression. However, in recent years, developments in mass

spectrometry (MS) based methodology has led to the widespread application of what is now referred to as quantitative targeted absolute proteomics.

This thesis presents the application of such a method for the determination of absolute protein content of CYP4F and CYP3A enzymes in human liver microsomes. First, a brief review of the various methodologies used to determine CYP expression and distribution is provided. Subsequently, absolute enzyme protein contents were determined using LC/MS/MS for a panel of 20 individual donor HLM samples and the inter-individual variability was characterized. The contribution of CYP4F enzymes to the overall hepatic CYP "pie" was also determined. The coherence of the LC/MS/MS method was evaluated by comparison to Western blot and enzyme activity results. Finally, chemical inhibition studies were performed to further investigate the contribution of CYP4F enzymes to the metabolic pathways of potential probe substrates. The work presented herein highlights the power of high-throughput LC/MS/MS-based methods for proteomics research through the accurate and reproducible determination of absolute protein contents for enzymes sharing extremely high (≥ 93%) sequence identity.

1.2 Chapter Summaries

1.2.1 Chapter Two: CYP Enzymes and Review of Analytical Methods

This chapter presents an overview of CYP enzymes with a focus on their physiological and clinical implications. An introduction to CYP4F and CYP3A enzymes is provided and examples of some representative metabolic pathways are described for each of the enzymes of interest. The remainder of the chapter presents a brief review of the analytical methodology that is available for the analysis of the expression levels of these membrane-associated proteins. The

methodologies are grouped into three major types, specifically mRNA-based, antibody-based, and LC/MS/MS-based methods.

1.2.2 Chapter Three: Determination of Enzyme Protein Content

This chapter describes the application of an LC/MS/MS analytical method for the absolute quantitation of enzyme protein content in individual donor HLM samples. The enzymes of interest were members of the CYP4F and CYP3A subfamilies, specifically CYP4F2, CYP4F3B, CYP3A4, and CYP3A5. Absolute enzyme protein concentrations were calculated relative to external standards, with linear calibration curves prepared using recombinant enzymes of known concentration. The recombinant enzyme standards and HLM samples were reduced, alkylated, and digested with trypsin. The resulting proteotypic peptides were used as selective markers for the proteins of interest to facilitate the quantitation absolute protein content in the HLM samples. Using this methodology, the absolute protein content and the inter-individual variability were elucidated for a panel of 20 individual donor HLM samples. The contribution of CYP4F enzymes to the overall hepatic CYP "pie" was also determined.

1.2.3 Chapter Four: Correlation of Protein Content and Enzyme Activity

This chapter is focused on the relationship between enzyme protein content and metabolic activity for each individual donor HLM sample. Quantitative LC/MS/MS methodology was developed to allow for the detection of metabolite formation following incubation of the HLM samples with probe substrates. The relationship between protein content and enzyme activity was then determined through correlation analysis. The identification of this relationship is critical in evaluating the utility of measuring protein content, as it is the functionality not the content of the enzymes present within the liver that dictate the anticipated

biological effect. The success of relating enzyme activity to protein content is dependent on the identification of probe substrates that possess a single metabolic elimination pathway that is specific for the enzyme of interest. Alternative routes of metabolism confound the elucidation of this relationship, as will be demonstrated for the CYP4F enzymes through chemical inhibition studies.

Chapter Two: CYP Enzymes and Review of Analytical Methods

2.1 Overview of CYP Enzymes

Cytochrome P450 (CYP) enzymes comprise a superfamily of proteins involved in the metabolism of numerous endogenous and exogenous substances. The human CYP genome contains 57 functional genes and 33 psuedogenes arranged into 18 families and 42 subfamilies (Nebert and Russell, 2002). The standard nomenclature assigns enzymes that share ≥ 40% sequence homology into a particular family which is indicated by an Arabic numeral. Subfamilies consist of enzymes sharing ≥ 55% sequence homology and are indicated by a letter. Individual members within a subfamily are indicated by a subsequent Arabic numeral. When first discovered, it was believed that the primary function of CYPs was to metabolize exogenous substances (e.g., drugs, pollutants, natural plant products) and that they were significantly expressed only in the liver. It is now well known that CYPs are also critically involved in biotransformation of numerous endogenous compounds (e.g., steroids, bile acids, pro-inflammatory substances) and are expressed in nearly every tissue, with appreciable concentrations found in the liver, small intestinal mucosa, lung, kidney, and brain (Paine et al., 2006).

Historically, much of the research into CYP-mediated drug disposition has focused on a relatively small subset of enzymes. It has been suggested that approximately 80 percent of oxidative drug metabolism, and almost 50 percent of the overall elimination of commonly used drugs, can be attributed to various members of the CYP1, CYP2, and CYP3 families. Within this small subset, CYP3A has received the most attention due to its abundant expression in major organs of elimination (Shimada et al., 1994; Paine et al., 2006) and its ability to metabolize a

wide range of structurally diverse substrates (Wilkinson, 2005). The increased attention with respect to many other drug metabolizing enzymes has resulted in the development of numerous analytical tools to characterize the expression and activity of CYP3A enzymes. Highly specific antibodies are widely available to allow for the immunoquantification of CYP3A enzymes and well characterized substrates, inducers, and inhibitors are available to study the role CYP3A enzymes may play in the disposition of a drug candidate.

Progress in the fields of genomics and proteomics has allowed for the identification and characterization of a wide variety of CYPs that had previously received little attention. Of particular importance has been the discovery and characterization of the CYP4 enzymes. This family of enzymes catalyzes the ω-hydroxylation of saturated, branched chain, and unsaturated fatty acids such as the physiologically important eicosanoids, prostaglandins, leukotrienes, and arachidonic acid. Within the CYP4 family, the CYP4F subfamily of enzymes has received significant attention for its ability modulate both pro- and anti-inflammatory mediators which suggests these enzymes may play a role in both the activation and resolution phase of the inflammatory response (Hardwick, 2008). Specifically, CYP4F enzymes have been shown to efficiently metabolize arachidonic acid to the potent pro-inflammatory modulator 20-HETE, a key step in the initiation of the inflammatory cascade that involves the production of numerous pro-inflammatory leukotrienes and prostaglandins. Additionally, metabolism by CYP4F enzymes has been identified as the main inactivation pathway of the pro-inflammatory mediator leukotriene B_4 (LTB_4). The inactivation of this potent pro-inflammatory eicosanoid suggests that CYP4F is also involved in the resolution phase of the inflammatory response (Sehgal et al., 2011).

In addition to their endogenous functions, CYP4F enzymes have been implicated in the metabolism of many commonly used drugs such as erythromycin, benzphetamine,

ethylmorphine, and ebastine (Jin et al., 2011). The presence of a genetic polymorphism in the CYP4F2 enzyme has been shown to significantly contribute to the individual variability of patient response upon administration of warfarin, which can have severe clinical implications due to the narrow therapeutic index of warfarin (McDonald et al., 2009). CYP4F2 and CYP4F3B have also been shown to be the major hepatic and enteric enzymes responsible for the initial *O*-demethylation of the antiparasitic prodrug parfuramidine (DB289) to form the primary metabolite M1 (DB775) (Wang et al., 2006; Wang et al., 2007). These recent developments suggest that the CYP4F enzymes, particularly CYP4F2 and CYP4F3B, play a significant role in the metabolism of both endogenous and exogenous substances.

Characterization of the location, expression, and functionality of these enzymes is critical to further understanding the roles they play in various physiological and pathological conditions. The remainder of this chapter will briefly discuss the analytical methods that are currently available for the quantitation of absolute enzyme protein content. The advantages and limitations associated with the various methods will be highlighted. The subsequent chapters present the quantitation of CYP4F and CYP3A protein contents and the corresponding relationship to enzyme activity.

2.2 Analytical Methodology for the Quantitation of Enzyme Protein Content

2.2.1 mRNA-based Methodology

The measurement of mRNA to study gene expression is a relatively well-established technique that has found wide application in molecular biology. The classical technique involving mRNA analysis is Northern blotting. The key steps involved in this technique are the separation of various mRNA using gel electrophoresis, transfer of the mRNA to a membrane, and finally crosslinking and hybridization with a labeled probe. This method is limited by low

sensitivity, large sample requirements, and problematic interpretation due to mRNA degradation during the process. However, the technique remains to be a gold standard for the direct study of gene expression at the mRNA level due to its simplicity and excellent specificity (Streit et al., 2009).

In addition to Northern blot analysis, the use of reverse transcription polymerase chain reaction (RT-PCR) has been applied to the quantitation of mRNA. This technique overcomes many of the limitations of Northern analysis, as the amplification of mRNA affected by the "chain reaction" results in adequate signal sensitivity for low abundance genes of interest (Sarikaya et al., 2006). In recent years, a technique referred to as *real-time* RT-PCR has emerged as a proven method for highly quantitative analysis of mRNA content. This approach applies fluorescent labeling techniques to allow for the measurement of the amplification products during each cycle of PCR. This method allows for the quantitation of various mRNA with great sensitivity and is amenable to high throughput formats, such as 96- and 384-well plates. The measurement at each stage of the amplification cycle overcomes the limitations of traditional RT-PCR, such as exponential growth during amplification that can reduce the accuracy of quantitation due to over-amplification (Girault et al., 2005).

The analysis of mRNA has proven to be an invaluable method of studying gene expression. However, extension of this methodology to investigate protein expression has encountered significant drawbacks. First, the relationship between mRNA content and protein expression levels has been shown to be highly variable. It has been proposed (Schwanhausser et al., 2011) that the correlation between mRNA and protein expression is likely to be better when the mRNA codes for stable proteins (i.e. metabolic genes) than for unstable proteins (i.e. cell signaling genes). Additionally, a variety of post-transcriptional controls affect the rates of protein synthesis such that up- or down-regulation can occur independent of mRNA levels.

Similarly, the activity of many proteins is affected by post-translational modifications such that up- or down-regulation of enzymatic activity may occur without significant changes in the absolute protein levels (Suarez and Moyes, 2012).

2.2.2 Antibody-based Methodology

Western blot analysis is a commonly used antibody-based technique for quantitation of protein content in various tissues or samples. The technique relies on a combination of a high-resolution separation, typically achieved by gel electrophoresis, and highly specific detection by immunochemical techniques. After separation of the various proteins in a sample by SDS-PAGE, the protein pattern is transferred onto a membrane and incubated with a specific antibody for the protein of interest (primary antibody). A second incubation is performed with a conjugated secondary antibody to allow for detection of the analyte protein using various methods (e.g. chemiluminescence, fluorescence, colorimetric, and autoradiography) depending on the type of the conjugated probe (Jansen et al., 1996).

Western blot analysis provides a highly sensitive method capable of absolute quantitation of proteins of interest. Antibodies typically display a very strong binding affinity and a high degree of specificity for their substrate, providing excellent sensitivity and selectivity for this technique when used to quantitate structurally diverse proteins. However, the situation becomes significantly more complicated when using Western blot analysis to quantitate proteins that share a high degree of sequence identity, such as the CYPs. Additionally, conventional approaches to antibody production are time consuming and can be inconsistent with respect to affinity and, particularly, specificity of the product (Edwards et al., 2003). Antibodies produced against intact proteins often cross-react with related proteins (i.e., enzymes of the same subfamily), preventing accurate individual quantitation of closely related proteins. Recently, methodology has been

proposed to overcome the issue of cross-reactivity during immunoquantification by developing antibodies that are specific for peptide sequences that are unique to the protein of interest. The protein content of CYP1A2 and CYP2E1 were accurately quantitated by this method, providing an orthogonal technique to confirm results obtained by traditional Western blot analysis (Kornilayev and Alterman, 2008).

2.2.3 LC/MS/MS-based Methodology

In recent years, mass spectrometry (MS) has emerged as the tool of choice for the accurate and specific quantitation of proteins in complex biological mixtures due to advances in the accuracy, sensitivity, and throughput capabilities of the methodology (Aebersold and Mann, 2003). The foundation of MS-based proteomics involves the identification of proteotypic peptides. These peptides are amino acid sequences unique to the protein of interest that result from enzymatic digestion (e.g., trypsin) and can be used as a marker for quantitation of the target protein.

The identification of the proteotypic peptide sequence can be predicted *in silico* using various protein sequence databases, though any selection should be confirmed through experimentation to ensure adequate abundance and ionization efficiency for the proposed peptide (Kawakami et al., 2011). Attempts have been made to develop selection criteria for proteotypic peptides (Kamiie et al., 2008). To facilitate detection by MS, it has been proposed that peptide length should be between 6 and 16 amino acids, possess no post-translational modification (PTM), and posses no single nucleotide polymorphisms (SNP). For stability of the peptide, no methionine or cysteine residues should be present to avoid complications due to oxidation. To reduce the likelihood of missed cleavages, the peptide should not contain sequential arginine or lysine residues in the digestion region and should not span any transmembrane regions. These

criteria were successfully applied in the quantitation of 11 CYP enzymes in HLM samples (Kawakami et al., 2011).

The typical workflow for LC/MS/MS-based methodology involves the solubilization of proteins from a biological sample, digestion of the protein samples by proteolytic enzymes, peptide separation by liquid chromatography (LC), and peptide detection by MS. If desired, the proteins can be separated by gel electrophoresis prior to digestion to reduce the complexity of the sample, though this significantly reduces the throughput of the method. Sample handling plays a critical role in the accuracy of quantitation by LC/MS/MS-based methods due to the reliance on peptide generation by enzymatic cleavage. Therefore, samples and standards should be digested in parallel and intact protein used as a standard, if possible, to account for non-stoichiometric digestion and varying protease activity (Wang et al., 2008).

The scientific literature contains numerous examples of the increasing application of LC/MS/MS-based methodology used to quantitate proteins of interest. The primary advantage of such techniques is that they are applicable to a wide variety of proteins, are not restricted to those which have well characterized antibodies to facilitate their study, and the specificity of MW-based methods is far superior to that of antibody-based methods for closely related proteins. Specifically, a series of 11 CYP enzymes commonly expressed in the human liver (CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, -3A4, -3A5, -3A43) were simultaneous quantitated in HLM samples using LC/MS/MS with *in silico* peptide selection (Kawakami et al., 2011). In addition to CYPs, LC/MS/MS-based proteomics has been applied to the absolute quantitation of various transporters such as the bile salt export pump, breast cancer resistance protein, and multidrug resistance-associated protein (Li et al., 2009a; Li et al., 2009b). The following chapter presents an application LC/MS/MS-based proteomics to quantitate CYP4F2 and CYP4F3B enzyme content in HLM samples. The enzymes were readily distinguished from one

another despite the ~93% sequence identity between the two proteins (Wang et al., 2006). The quantitative analysis of CYP3A enzyme content by LC/MS/MS is also presented. The absolute enzyme protein contents determined by LC/MS/MS were cross-validated by immunoquantification. The final chapter describes the use of probe substrates to investigate the relationship between protein content and enzyme activity.

Chapter Three: Determination of CYP4F and CYP3A Protein Content

3.1 Introduction

The recent identification of the role CYP4F enzymes, specifically CYP4F2 and CYP4F3B, play in drug metabolism has resulted in the need for analytical methodology that can accurately quantitate CYP4F protein levels in various tissues. Additionally, characterization of the inter-individual variability of protein expression will help inform dosage optimization in clinical settings involving CYP4F substrates, inhibitors, or inducers.

Traditional methods of protein quantitation have relied on antibodies that are highly specific for the protein of interest; however, this methodology is prone to failure when trying to distinguish between enzymes such as CYP4F2 and CYP4F3B which share approximately 93% sequence identity (Wang et al., 2006). Recent advances in LC/MS/MS methodology have circumvented the issue of specificity by utilizing "proteotypic" peptides resulting from an enzymatic digestion for the detection of proteins of interest. Based on the amino acid sequence of the target protein and the known cleavage sites of the digesting enzyme, peptide sequences can be identified that are specific for the protein of interest. In addition to sequence exclusivity, proteotypic peptides must be highly abundant or display adequate ionization behavior to allow for detection by mass spectrometry (Langenfeld et al., 2008). Due to the large number of peptides that result from the enzymatic digestion of complex biological samples, chromatographic separation of peptide fragments is employed to reduce the potential for ion suppression and improve the sensitivity and specificity of the analytical method.

The objective of the study presented herein was to optimize and apply an LC/MS/MS-based proteomics method to the quantitative analysis of CYP4F and CYP3A protein content in a

series of 20 individual donor HLM samples. The MS-based protein contents were correlated to immunoquantified protein contents to evaluate the coherence of the orthogonal analytical methods. This work is intended to quantitatively evaluate the absolute protein content of CYP4F enzymes in the human liver for the first time and describes their contribution towards the overall hepatic CYP enzyme "pie" that was first determined by Shimada et al (1994). The identification of inter-individual variability in the protein expression levels of the CYP4F enzymes is critical to understanding the factors that affect the safety, disposition, and efficacy of their therapeutic substrates. The evaluation of CYP3A enzyme expression and activity was included as a measure of the method performance, due to the well characterized analytical tools available for these enzymes.

3.2 Materials and Methods

3.2.1 Chemicals

Sequencing-grade modified trypsin and NADPH regenerating solution were purchased from Promega (Madison, WI). HPLC-grade acetonitrile (ACN) and methanol were obtained from Fisher Scientific (Morris Plains, NJ). Analytical-grade acetic acid, ammonium acetate, ammonium bicarbonate, dithiothreitol (DTT), formic acid, iodoacetamide (IAA), potassium phosphate, and other reagents were purchased from VWR (Bridgeport, NJ). Reagents were of the highest purity available and used without further purification.

3.2.2 Human Liver Microsomes, Recombinant Human CYP Enzymes, and Antibodies

Non-fetal (age 6 - 67) individual donor HLMs (n=20; H0422, H0425, H0428, H0430, H0431, H0432, H0441, H0444, H0450, H0451, H0452, H0453, H0459, H0472, H0482, H0715,

H0728, H0737, H0743, H0802) were obtained from XenoTech, LLC (Lenexa, KS) and consisted of both male (n=12) and female (n=8) donors. Pooled HLM (n=200; XTreme 200, lot# 1010420) was also purchased from XenoTech, LLC. Recombinant CYP3A4, CYP3A5, CYP4F2, and CYP4F3B Supersomes™ prepared from baculovirus-infected insect cells expressing human CYP enzymes and NADPH-cytochrome P450 reductase were purchased from BD Gentest (Woburn, MA). CYP4F2 and CYP4F3B Supersomes™ also coexpressed cytochrome b_5. Total CYP concentration (pmol/mL) in each Supersomes™, determined by a spectrophotometric method (Omura and Sato, 1963), was provided by the supplier. A mouse monoclonal anti-CYP3A antibody (WB-MAB-3A, Lot 30494; detects human CYP3A4, CYP3A5, and CYP3A7) was purchased from BD Gentest. A polyclonal antibody raised against CYP4F2 was purchased from Research Diagnostics, Inc. (Concord, MA) (1 mg IgG/mL).

3.2.3 Synthetic Proteotypic Peptides

Synthetic unlabeled and ^{13}C-, ^{15}N-labeled proteotypic peptides were ordered from Thermo Scientific (Ulm, Germany). The isotope-labeled peptides were used as the internal standard (IS) during the quantitative analysis of peptide content in HLM samples. The sequence information for each proteotypic peptide used in the work presented herein is summarized in Table 1. Peptide purity (>94%) was provided by the manufacturer, using RP-HPLC UV (detection wavelength at 215 nm) analysis and MALDI-TOF MS analysis. Peptide identity was confirmed by obtaining product ion spectra via infusion into an API4000 QTrap (MDS Sciex, Framingham, MA) triple quadrupole mass spectrometer.

Table 1. Sequence information and ionization parameters for proteotypic peptides

CYP	Peptide Name	Proteotypic Peptide Sequence	M+H	MRM (m/z)
CYP3A[a)]	3A_pep1	S_{131}LLSPTFTSGK$_{141}$	1138	569.6 → 737.6
	3A_IS_pep1[b)]	S_{131}LLSPT**F**(9 ^{13}C, ^{15}N)TSGK$_{141}$	1148	574.4 → 834.5
CYP3A4	3A4_pep1	E_{244}VTNFLR$_{250}$	879	439.9 → 650.5
	3A4_IS_pep1	E_{244}VTN**F**(9 ^{13}C, ^{15}N)LR$_{250}$	889	444.8 → 660.5
CYP3A5	3A5_pep1	D_{244}TINFLSK$_{251}$	938	469.5 → 608.4
	3A5_IS_pep1	D_{244}TIN**F**(9 ^{13}C, ^{15}N)LSK$_{251}$	948	474.4 → 618.5
CYP4F2	4F2_pep1	S_{109}VINASAAIAPK$_{120}$	1142	571.6 → 842.6
	4F2_IS_pep1	S_{109}V**I**(6 ^{13}C, ^{15}N)NASAAIAPK$_{120}$	1149	575.0 → 962.6
	4F2_pep2	V_{480}VLALTLLR$_{488}$	998	499.6 → 799.7
	4F2_IS_pep2	V_{480}V**L**(6 ^{13}C, ^{15}N)ALTLLR$_{488}$	1005	503.0 → 806.7
CYP4F3B	4F3B_pep1	S_{109}VINASAAIVPK$_{120}$	1170	585.6 → 870.6
	4F3B_IS_pep1	S_{109}V**I**(6 ^{13}C, ^{15}N)NASAAIVPK$_{120}$	1177	589.0 → 870.6
	4F3B_pep2	V_{480}VLGLTLLR$_{488}$	984	492.5 → 785.6
	4F3B_IS_pep2	V_{480}V**L**(6 ^{13}C, ^{15}N)GLTLLR$_{488}$	991	496.0 → 792.6

a) This peptide detects CYP3A4, CYP3A5, and CYP3A7, but not CYP3A43.
b) The ^{13}C- and ^{15}N-labeled peptides were used as internal standards (IS) during quantitation.

3.2.4 Optimization of MS-parameters for Proteotypic Peptides

Solutions of synthetic proteotypic peptides were prepared in 50:50 ACN:1% acetic acid to a final concentration of ~1 μM. The solutions were infused (0.01 mL/min), along with a supplementary mobile phase flow (0.4 mg/mL; 50% ACN, 0.1% formic acid, v/v), into an Applied Biosystems API4000 QTrap triple quadrupole mass spectrometer equipped with a Turbo IonSpray® interface (MDS Sciex). Ionspray voltage and turbo heater temperature were maintained at 2500 V and 500 °C, respectively. Product ion (MS/MS) spectrum for each proteotypic peptide was acquired on the doubly charged precursor ion. Ion fragments yielding the best sensitivity were selected as the basis for the multiple reaction monitoring (MRM) transitions used for quantitation of the peptides. Compound specific instrument parameters were

optimized for each transition to obtain the most abundant signal and are summarized in Table 1. The MS instrument was operated under positive ion mode and unit resolution for both Q1 and Q3.

3.2.5 Protein Digestion and Quantitation by LC/MS/MS

Digestion of recombinant CYPs and HLMs was performed according to a modified version of the procedure developed previously (Wang et al., 2008). The final protocol involved the following steps: reduction of protein samples (10 - 30 μg) in a buffer (90 μL) containing 50 mM ammonium bicarbonate (pH 8) and 4 mM DTT. The mixture was then heated at 60°C for 60 min to denature the proteins and allowed to cool to room temperature. The reduced and denatured samples were alkylated by IAA at a final concentration of 10 mM for 20 min in the dark. Upon completion of the alkylation reaction, 1 μg of trypsin was added to the protein samples, and digestion was carried out at 37°C for 4 h. Samples were shaken briefly approximately midway through the digestion. The digestion reactions were terminated by the addition of half-volume of ice-cold ACN containing ^{13}C-, ^{15}N-labeled proteotypic peptides as internal standards. The samples were mixed by vortex and precipitated protein was removed by centrifugation at ~4000 rpm for 10 minutes. Supernatants were transferred to clean 96-well plates and 8 μL was injected for LC/MS/MS analysis. Chromatographic separation of proteotypic peptides was obtained as described previously (Wang et al., 2008).

3.2.6 Preparation of Calibration Standards

Linear calibration standards were prepared from various concentrations of recombinant CYPs (0.01 - 10 pmol). Standards and samples were digested in parallel and the proteotypic peptides resulting from protein trypsinolysis were used to generate calibration curves.

Quantitation was performed using the ratio of proteotypic peptide peak area to the corresponding isotopically enriched peptide peak, which was present at constant levels as the internal standard.

3.2.7 Data Analysis

Data for quantitation of enzyme protein content are presented as the mean of at least triplicate determinations, unless indicated otherwise. Total CYP4F content was determined as the sum of individual data points calculated using "_pep1" and "_pep2". The contribution of CYP4F to the human liver CYP "pie" was determined by assuming the total CYP3A enzyme content determined in this study represented the same percentage of the CYP "pie" originally determined for CYP3A by Shimada et al (1994). This value permitted direct correlation of the observed CYP4F enzyme content to a percentage of the "pie", which was assumed to represent a portion of the previously unknown enzyme content. For least-squares linear regression analysis, the coefficient of determination (R^2) was determined using the Prism GraphPad (Version 6.0, San Diego, CA) software; a p value < 0.05 was considered significant..

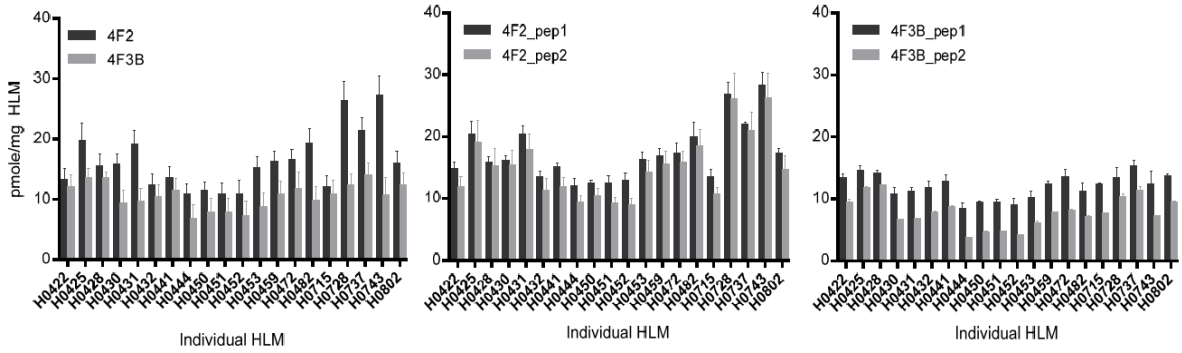
3.3 Results and Discussion

3.3.1 Quantitation of Absolute CYP4F Protein Content in HLM

The absolute enzyme protein content was determined for CYP4F2 and CYP4F3B in 20 individual donor HLM samples. Two unique proteotypic peptides were identified for each enzyme and used for quantitation of protein expression levels. The average (range) CYP4F2 protein content for the individual donor samples was calculated to be 17.0 (11.9 - 28.1) and 14.9 (8.8 - 26.1) pmole/mg HLM using 4F2_pep1 and 4F2_pep2, respectively. The average CV was observed to be 8% and 15% using 4F2_pep1 and 4F2_pep2, respectively. These results

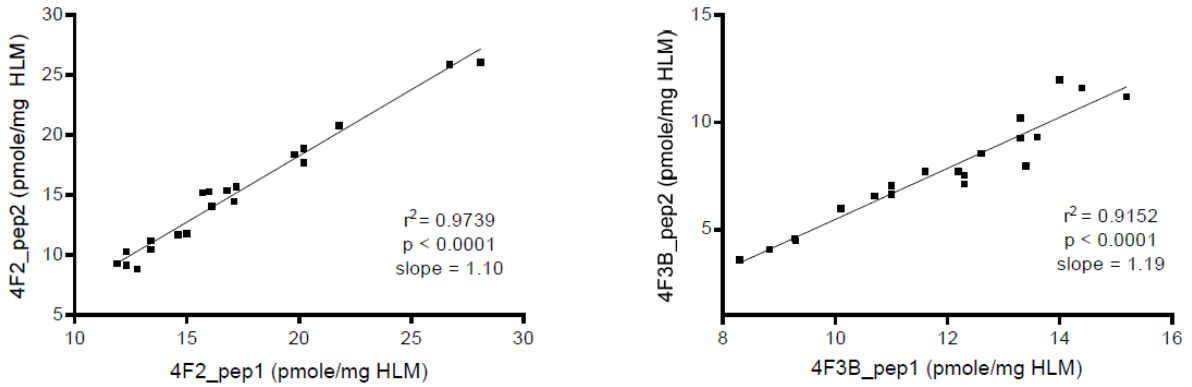
correspond to a global average (range) of CYP4F2 protein content for the individual donor samples of 16.1 (10.7 – 27.1) pmole/mg HLM and an average CV of 14%. The average (range) CYP4F3B protein content for the individual donor samples was calculated to be 11.9 (8.3 - 15.2) and 7.6 (3.6 - 12.0) pmole/mg HLM using 4F3B_pep1 and 4F3B_pep2. The average CV was observed to be 9% and 4% using 4F3B_pep1 and 4F3B_pep2, respectively. These results correspond to a global average (range) of protein content for CYP4F3B in the first panel of HLM of 10.4 (6.7 – 13.9) pmole/mg HLM and an average CV of 23%. The results for quantitation of CYP4F protein content are summarized in Figure 1.

Figure 1. Absolute CYP4F protein content in individual donor HLM



The inter-individual variability for CYP4F2 protein content was observed to be 2.4- and 3.0-fold when calculated using 4F2_pep1 and 4F2_pep2, respectively. The inter-individual variability for CYP4F3B protein content was observed to be 1.8- and 3.4-fold when calculated using 4F3B_pep1 and 4F3B_pep2, respectively. The enzyme protein content determined for the individual donor HLM samples using the two unique proteotypic peptides showed a strong correlation for both CYP4F2 (r^2=0.97, $p < 0.0001$) and CYP4F3B (r^2=0.92, $p < 0.0001$), as shown in Figure 2.

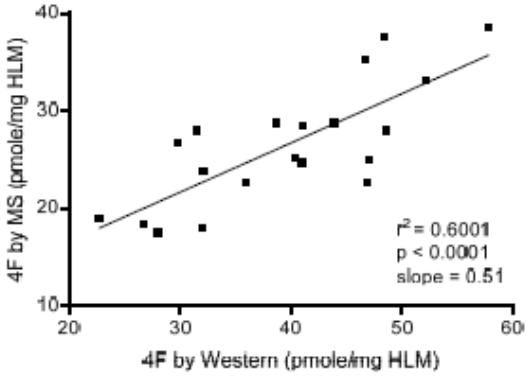
Figure 2. Correlation of CYP4F protein content calculated by multiple proteotypic peptides



3.3.2 Cross-Validation of LC/MS/MS Method with Immunoquantification for CYP4F

Western blot analysis was performed on the individual donor HLM samples to quantitate absolute CYP4F protein content by an orthogonal technique. The samples were immunoblotted with an antibody raised against CYP4F. This antibody is thought to detect CYP4F2 and CYP4F3B, but not CYP4F12. Reactivity towards additional CYP4F enzymes has not been quantified at this time. Therefore, the total CYP4F protein content measured by LC/MS/MS, determined as the sum of the average CYP4F2 and the average CYP4F3B protein contents, was used for comparison with immunoquantified results. The average (range) CYP4F protein content for the individual donor HLM samples was calculated to be 26.4 (17.5 - 38.5) and 39.6 (22.7 - 57.9) pmole/mg HLM when determined by LC/MS/MS and immunoquantification, respectively. The enzyme protein content values determined by Western blot analysis were consistently higher than those determined by LC/MS/MS. This difference in protein content may be the result of the fact that the antibody specificity towards additional hepatic CYP4F enzymes, specifically CYP4F11, is unknown at this time. Given the differences in enzyme specificity, the two methods were well correlated ($r^2 = 0.60$, $p < 0.0001$) as shown in Figure 3.

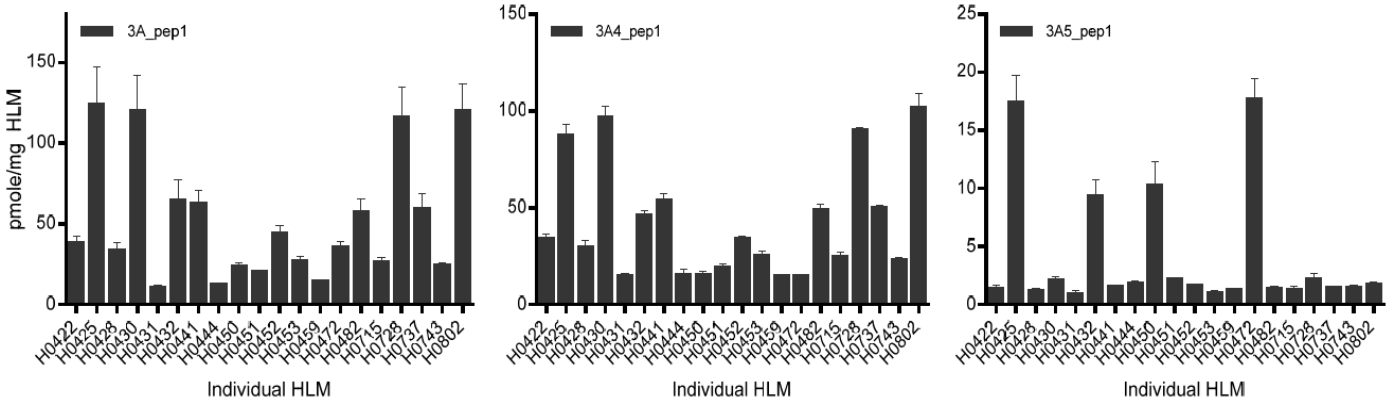
Figure 3. Correlation of CYP4F protein content calculated by orthogonal techniques



3.3.3 Quantitation of Absolute CYP3A Protein Content in HLM

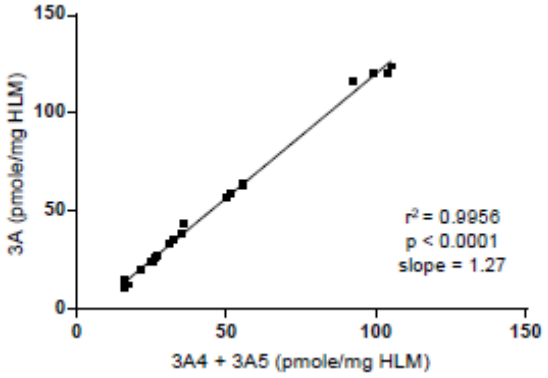
The absolute enzyme protein content was determined for CYP3A, CYP3A4, and CYP3A5 in 20 individual donor HLM samples. The proteotypic peptide used to quantify total CYP3A includes the enzymes CYP3A4, CYP3A5, and CYP3A7, but not CYP3A43. The proteotypic peptides used to quantify CYP3A4 and CYP3A5 were confirmed to contain amino acid sequences unique to each enzyme, as described previously (Wang et al., 2006). The average (range) protein content for CYP3A, CYP3A4, and CYP3A5 was observed to be 52.5 (10.8 - 124), 42.2 (14.6 - 102), and 4.0 (0.89 - 18) pmole/mg HLM, respectively. The average CV was observed to be 12%, 8%, and 14% for 3A_pep1, 3A4_pep1, and 3A5_pep1, respectively. These results are summarized in Figure 4.

Figure 4. Absolute CYP3A protein content in individual donor HLM



CYP3A5 was expressed at the lowest levels but showed the greatest inter-individual variability in enzyme protein content, with a 20-fold difference between the individual donor with the highest expression level and the donor with the lowest. The inter-individual variability in CYP3A and CYP3A4 were significantly lower, with an 11- and 7-fold difference between the donors with the highest and the lowest expression level, respectively. This magnitude of variability is significantly greater than that observed for the CYP4F enzymes. In the human liver, the expression of CYP3A4 and CYP3A5 accounts for the majority of CYP3A protein in the human liver. Therefore, the CYP3A protein content (as calculated by the 3A_pep1) was plotted against the sum of CYP3A4 and CYP3A5 (as calculated by 3A4_pep1 and 3A5_pep1, respectively). Strong correlation ($r^2 = 0.996$, $p < 0.0001$) was observed confirming the coherence of the LC/MS/MS methodology, shown in Figure 5.

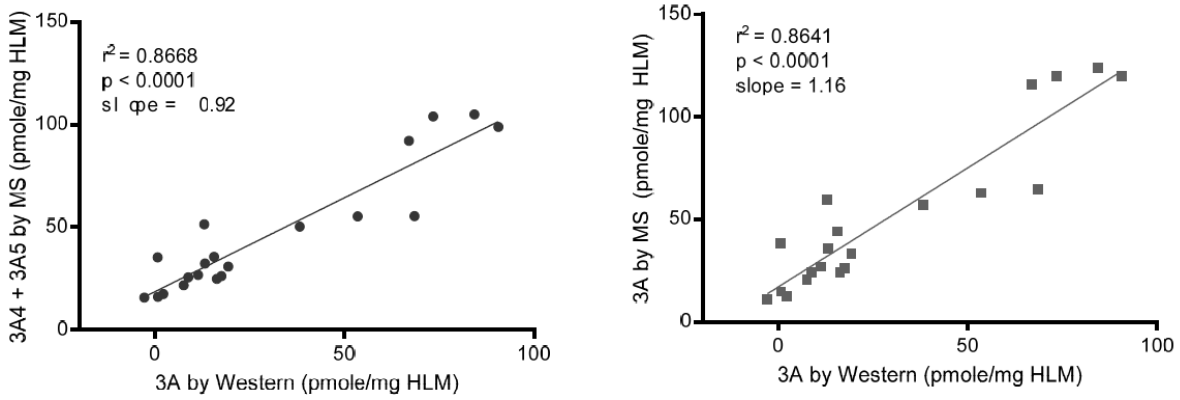
Figure 5. Correlation of CYP3A protein content calculated by multiple proteotypic peptides



3.3.4 Cross-Validation of LC/MS/MS Method with Immunoquantification for CYP3A

Western blot analysis was performed on the individual donor HLM samples to quantitate absolute CYP3A protein content by an orthogonal technique. The samples were immunoblotted with a mouse anti-CYP3A antibody that detects human CYP3A4, CYP3A5, and CYP3A7. The average (range) CYP3A protein content for the individual donor HLM samples was calculated to be 30.2 (0 - 90.6) pmole/mg HLM when determined by immunoquantification. Contrary to CYP4F, the CYP3A protein content determined by Western blot analysis were consistently lower than those determined by LC/MS/MS, though a strong correlation was observed for the two methods when using both the CYP3A-specific peptide (r^2=0.86, $p < 0.0001$) and the sum of the CYP3A4- and CYP3A5-specific peptides (r^2=0.87, $p < 0.0001$). These results are summarized in Figure 6.

Figure 6. Correlation of CYP3A protein content calculated by orthogonal techniques



3.4 Summary

This chapter presents the application of a rapid and robust LC/MS/MS analytical method to determine the absolute protein content of various CYP4F and CYP3A enzymes in individual donor HLM samples. The method was cross-validated by the orthogonal technique of quantitative Western blot analysis and good correlation ($r^2 \geq 0.60$) was observed for all enzymes of interest. The ability to quantitate absolute protein content without the need for production of antibodies is highly attractive for several reasons, some of which are outlined below. The cost and time associated with developing antibodies with the requisite specificity for accurate quantitation of homologous proteins is quite significant. Additionally, the use of antibody-based assays is not amenable to the high-throughput screening that is desired from a drug development perspective. Therefore, the use of LC/MS/MS techniques for quantitative proteomics has received significant attention in recent years.

The current work applied such a method to a panel of 20 individual donor HLM samples in which the absolute protein content and the inter-individual variability of specific enzymes was evaluated. It was observed that the CYP4F enzymes were expressed to a lesser degree and displayed significantly less (~2- to 4-fold) variability than did the CYP3A enzymes (~7- to 20-fold). These values for CYP4F are consistent with previous estimates of individual variability

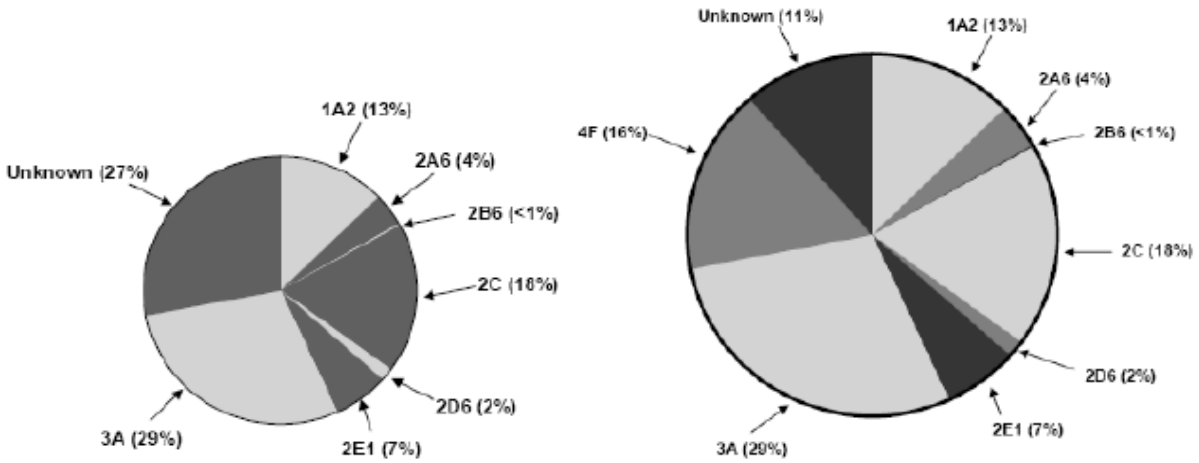
based on the observed rate of leukotriene B_4 20-hydroxylation (Jin et al., 1998), which suggested the variability to be approximately 3-fold amongst individuals. It has been estimated that the constitutive variability in CYP3A enzymes is about 5-fold, but that this variability can be increased to as high as 400-fold through factors such as co-administration of inhibitors or inducers, environmental factors, or various disease states (Wilkinson, 2005). The CYP3A variability observed in the current panel of HLM samples is consistent with this magnitude of variability.

It was interesting to note that for the CYP4F enzymes, the proteotypic peptide designated "_pep2" resulted in lower calculated protein contents for both CYP4F2 (average of 13% lower) and CYP4F3B (average 37% lower). The differences in calculated protein contents were determined to be statistically insignificant for CYP4F2; however, the differences for CYP4F3B protein content were determined to be statistically significant ($\alpha = 0.05$) using the unpaired t-test. The reasons for this discrepancy are not clear at this time. Upon review of the National Institute of Standards and Technology (NIST) website for peptide fragment spectra (http://peptide.nist.gov/), it was observed that only the "_pep1" sequence is identified as a proteotypic peptide fragment following digesting. However, the unique amino acid sequence for "_pep2" is preceded by a lysine and is terminated by a arginine, suggesting it should be cleaved by tryptic digest. Additionally, the use of intact recombinant protein as the calibration standards should also help guard against incomplete digestion due to preferential attack by the enzyme at one cleavage site versus the other. Additional investigation is required to identify the underlying causes for the bias in protein contents calculated by "_pep1" and "_pep2", which is outside the scope of this thesis.

The results presented herein suggest that the CYP4F family of enzymes represent a significant portion of the overall human liver CYP “pie”. It was previously shown that

approximately 70% of the total human liver CYP content could be accounted for by CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2D6, CYP2E1, and CYP3A with the remaining 30% constituting then unknown enzymes (Shimada et al., 1994). Based on the results of this work, we propose that a significant portion (~16%) of the previously unknown enzyme content is accounted for by CYP4F enzymes which makes them the third largest piece of the human liver "pie" and strengthens the hypothesis that these enzymes play an important role in the metabolism of endogenous and exogenous substances in the human body. The calculation of the contribution of CYP4F to the overall pie was based on the assignment that the CYP3A content calculated in the current study corresponded to the percentage of the "pie" originally assigned by Shimada et. al (1994). This allowed the direct correlation of CYP4F protein content calculated in the current work (as pmole/mg HLM) to a percentage of the total protein content determined previously. This percentage was then subtracted from the previously unknown portion of the "pie" to generate the CYP4F contribution and the remaining unknown contribution. The original "pie" determined by Shimada et al (1994) and the revised "pie" incorporating the CYP4F content are shown in Figure 7.

Figure 7. Contribution of CYP4F to hepatic CYP "pie"



In addition to the liver, CYP4F enzymes have been identified as the major enteric enzymes that are responsible for DB289 M1 formation, indicating that there is significant expression of these enzymes in the intestine (Wang et al., 2007). However, no quantitative analysis has been performed to suggest how CYP4F content in the intestine contributes to the overall enteric CYP "pie". Further study is required to understand the significance of CYP4F expression in the intestine and how this may affect factors such as first-pass metabolism of therapeutic agents or the maintenance of intestinal homeostasis through the metabolism of various endogenous substances.

Chapter Four: Correlation of Protein Content and Enzyme Activity

4.1 Introduction

The absolute quantitation of protein content for a wide variety of drug metabolizing and drug transporting proteins is a relatively new practice. Prior to the availability of the advanced instrumentation which makes this absolute quantitation possible, the routine methods for assessing the content of a particular enzyme within a complex sample (e.g., microsomes) relied on the use of specific antibodies or probe substrates for the enzyme of interest. These methods are quite useful when antibodies or substrates are well-characterized and validated. However, cross-reaction of antibodies and promiscuity of substrates are often of concern, especially for newly identified proteins. A concern that is often encountered in a clinical setting is the potential for drug-drug interactions due to inhibition or induction of CYPs during co-administration of pharmaceuticals. A wide variety of marker reactions have been characterized and validated to screen for inhibition and/or induction of common drug metabolizing enzymes (Walsky and Obach, 2004) and this information is used to guide drug development programs.

The use of marker activities as a surrogate for protein quantitation has yielded invaluable insight into the distribution and expression levels of various enzymes (Iwatsubo et al., 1997; Ito et al., 1998). However, these methods are confounded by several issues. First, the specificity of a particular substrate/inhibitor/inducer is rarely such that only a single enzyme is involved in the reaction. Second, the availability of a specific substrate/inhibitor/inducer is quite limited for drug transporters and less commonly studied CYPs (Ohtsuki et al., 2012). However, the use of marker activities has remained common practice in drug development due to the wide

availability of reagents, simplistic experimental procedures, and amenability to high-throughput screening.

This chapter presents the correlation of the absolute CYP protein contents determined in the previous chapter with various marker activities for the enzymes of interest. The manufacturer-provided activities for CYP3A enzymes were confirmed through experiment to ensure that the integrity of the enzymes remained intact over the course of processing and shipping the microsome samples. Additionally, the antiparasitic prodrug parfuramidine (DB289) and a closely related structural analog (DB1230) were evaluated for the use as a probe of CYP4F activity. Enzyme inhibition experiments were then performed to further investigate the metabolic pathways involved in the biotransformation of DB289 and DB1230.

4.2 Materials and Methods

4.2.1 Chemicals

Parfuramidine (DB289), M1 (DB775), deuterium-labeled DB289 (DB289-d_8; internal standard), and a structural analog of parfuramidine (DB1230) were kindly provided by the Consortium for Parasitic Drug Development (CPDD), the University of North Carolina at Chapel Hill (Chapel Hill, NC). HET0016 was purchased from Cayman Chemical Co. (Ann Arbor, MI). NADPH regenerating solution was purchased from Promega (Madison, WI). Fluvoxamine was purchased from Toronto Research Chemicals (Toronto, ON). Midazolam and 6',7'-dihydroxybergamottin (DHB) were purchased from Cerillant (Round Rock, TX). Isotope-labeled ($^{13}C_3$) 1'-hydroxymidazolam was purchased from BD Biosciences (San Jose, CA). Azamulin was purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile (ACN) and methanol were obtained from Fisher Scientific (Morris Plains, NJ). Analytical-grade

dimethylsulfoxide (DMSO), formic acid, potassium phosphate, and other reagents were purchased from VWR (Bridgeport, NJ).

4.2.2 Human Liver Microsomes and Recombinant Human CYP Enzymes

Non-fetal (age 6 - 67) individual donor HLMs (n=20; H0422, H0425, H0428, H0430, H0431, H0432, H0441, H0444, H0450, H0451, H0452, H0453, H0459, H0472, H0482, H0715, H0728, H0737, H0743, H0802) were obtained from XenoTech, LLC and consisted of both male (n=12) and female (n=8) donors. Pooled HLM (n=200; XTreme 200, lot# 1010420) was also purchased from XenoTech, LLC. Recombinant CYP1A2, CYP3A4, CYP4F2, CYP4F3B, and CYP2J2 Supersomes™ prepared from baculovirus-infected insect cells expressing human CYP enzymes and NADPH-cytochrome P450 reductase were purchased from BD Gentest (Woburn, MA). CYP4F2, CYP4F3B, and CYP2J2 Supersomes™ also coexpressed cytochrome b_5. Total CYP concentration (pmol/mL) in each Supersomes™, determined by a spectrophotometric method (Omura and Sato, 1963), was provided by the supplier.

4.2.3 Marker Activities and Correlation Analysis

Testosterone 6β-hydroxylation and midazolam 1'-hydroxylation activities for the panel of HLM were provided by XenoTech. Midazolam 1'-hydroxylation activity was evaluated as described previously (Wang et al., 2007) to confirm the manufacturer provided values and ensure sample integrity following processing and delivery of HLM samples. DB289 primary metabolite formation (M1 formation), a marker for CYP4F activity (Wang et al., 2007), was measured as described previously. DB1230 primary metabolite formation (M1, *O*-demethylation product), a potential marker for CYP4F activity was also evaluated. Briefly, incubation mixtures contained 100 mM phosphate buffer (pH 7.4), 3.3 mM MgCl2, and 1 mM NADPH, unless indicated

otherwise. Reactions were initiated with the addition of NADPH following pre-incubation of the incubation mixture at 37 °C for 5 min. DB289, DB1230, and DB289-d_8 were dissolved in DMSO. Midazolam and $^{13}C_3$-1'hydroxymidazolam were dissolved in methanol. Substrates were present at 3 μM final concentration, except DB1230 which was present at 1 μM final concentration. All incubations contained 0.2 mg/mL HLM. Final incubation mixtures contained less than 1% (v/v) organic solvent. Reactions were terminated with the addition of equal volume of ice-cold ACN containing 0.1% formic acid and internal standard (either 20 nM DB289-d8 or 1 μM $^{13}C_3$-1'hydroxymidazolam). The mixtures were vortex-mixed and precipitated protein was removed by centrifugation at ~4000 rpm for 15 min. The resulting supernatants were analyzed by LC/MS/MS (described below).

4.2.4 Chemical Inhibition Assays

CYP-selective chemical inhibitors were added to standard incubation mixtures (200 μL) containing 0.2 mg/mL HLM or 10 pmole/mL recombinant CYP enzyme and 3 μM DB289 or 1 μM DB1230. The chemical inhibitors used were fluvoxamine (3 μM; CYP1A2 inhibitor), HET0016 (0.1 μM; an arachidonic acid ω-hydroxylase inhibitor), DHB (30 μM; CYP3A inhibitor), and azamulin (3 μM; CYP3A inhibitor). A double inhibition experiment was performed in individual donor HLM samples for DB289 in which both fluvoxamine (3 μM) and azamulin (3 μM) were included in the same incubation. At the end of the incubation, samples were processed as described above. Control incubations contained methanol in place of chemical inhibitors.

4.2.5 LC/MS/MS Analysis

Substrates and primary metabolites used for evaluation of marker reaction activity were quantitated using an Applied Biosystems API 4000 QTrap triple quadrupole mass spectrometer equipped with a Turbo IonSpray® interface (MDS Sciex) running Analyst software (Version 1.4.2). Samples were injected into the mass spectrometer using a cooled (10°C) CTC PAL LEAP autosampler (LEAP Technologies, Carrboro, NC), a Shimadzu LC-20 HPLC system (Shimadzu, Columbia, MD), and a Valco solvent divert valve (Valco, Houston, TX). Chromatographic separation was obtained using an Agilent HPLC column (Zorbax SB-C8, 2.1 x 50 mm, 3.5 µm; Agilent, Santa Clara, CA). HPLC mobile phase (A) consisted of 2 mM ammonium acetate, 0.1% formic acid in 95:5 (v/v) water:methanol and (B) consisted of 2 mM ammonium acetate, 0.1% formic acid in 5:95 (v/v) water:methanol. A 4 minute gradient elution method was employed; initial conditions of 40% B were held for 0.5 minutes and mobile phase B was increased to 95% over 1.5 minutes. These conditions were held for 2 minutes after which the column was re-equilibrated with mobile phase consisting of 40% B for 0.5 minutes prior to injecting the next sample.

4.2.6 Data Analysis

Data are presented as the mean of duplicate determinations unless indicated otherwise. For inhibition studies, the amount of metabolite formed in control incubations (no inhibitors) was considered 100%, to which the amount of metabolite formed in the presence of chemical inhibitors was normalized. The coefficient of determination (R^2) was determined by least-squares linear regression (Prism 6.0, GraphPad Software, San Diego, CA); a p-value < 0.05 was considered significant. Activity-adjusted CYP4F protein content was calculated by scaling the

individual CYP4F2 and CYP4F3B enzyme content according each enzymes relative activity towards DB289 and DB2130, as determined in recombinant systems.

4.3 Results and Discussion

4.3.1 Correlation of CYP Protein Content and Enzyme Activity

The rates of primary metabolite formation (*O*-demethylation) for the antiparasitic prodrug parfuramidine (DB289) and a closely related structural analog (DB1230) were determined for 20 individual donor HLM samples. Previous studies have suggested that CYP4F enzymes, specifically CYP4F2 and CYP4F3B, are the primary hepatic and enteric enzymes responsible for the initial *O*-demethylation of DB289. Several additional CYP enzymes, particularly CYP1A1, CYP1A2, CYP1B1, and CYP2J2, showed activity towards DB289 primary metabolite formation. CYP3A4 also showed activity, but was rather inefficient at metabolizing DB289 when compared to the other enzymes. However, previous chemical inhibition studies suggested while these enzymes displayed activity in recombinant systems, they did not play a significant role in the biotransformation of DB289 by HLM samples (Wang et al., 2006; Wang et al., 2007).

In the current study, the observed rates of DB289 and DB1230 metabolite formation were plotted versus the total CYP4F protein content determined by LC/MS/MS and immunoquantification. No correlation was obtained in either system studied. With respect to DB289, no correlation was observed for metabolite formation and CYP4F protein content as determined by LC/MS/MS (r^2=0.022, p = 0.53) or quantitative Western blot (r^2=0.044, p = 0.37), as shown in Figure 8. With respect to DB1230, no correlation was observed for metabolite formation and CYP4F protein content as determined by LC/MS/MS (r^2=0.079, p = 0.23) or quantitative Western blot (r^2=0.056, p = 0.32), as shown in Figure 9. Attempts to use the

activity-adjusted CYP4F protein content, which takes into account the differing activities of both CYP4F2 and CYP4F3B towards the substrate, did not result in an improved correlation with either DB289 (r^2=0.026, p = 0.49) or DB1230 (r^2=0.078, p = 0.23) primary metabolite formation (data not shown).

Figure 8. Correlation of CYP4F protein content and DB289 metabolite formation.

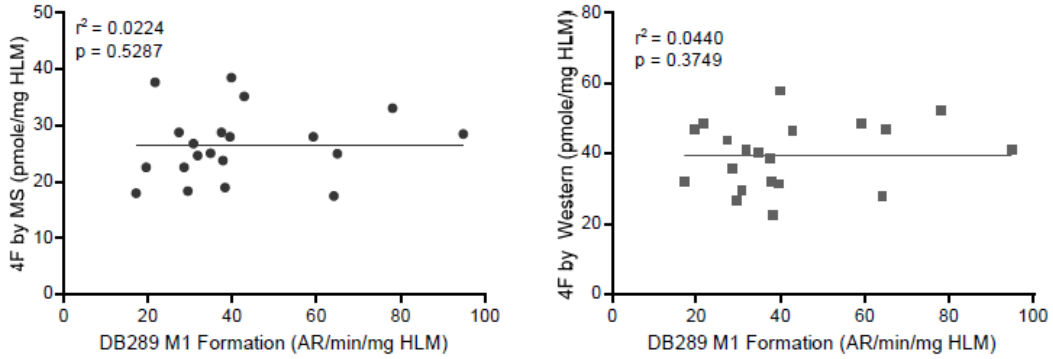
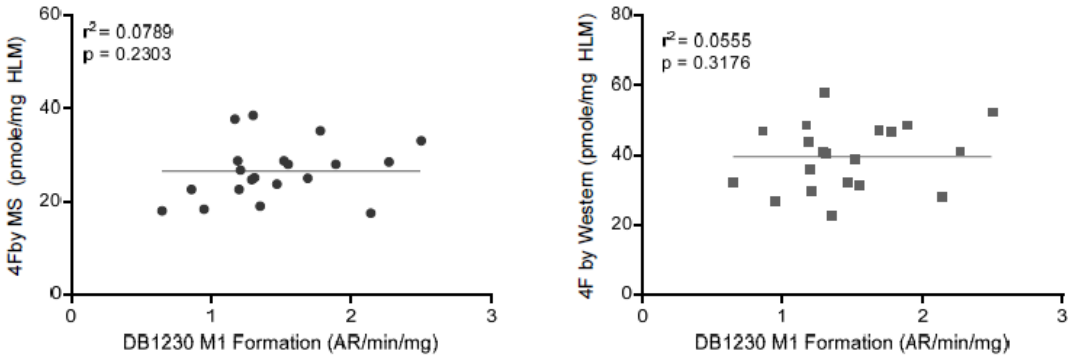


Figure 9. Correlation of CYP4F protein content and DB1230 metabolite formation.



The CYP3A protein content calculated in the previous chapter was plotted against various measures of enzyme activity to evaluate the ability of the LC/MS/MS methodology described herein to predict biological activity based on absolute enzyme protein content. Testosterone 6β-hydroxylation and midazolam 1'-hydroxylation activities were provided by XenoTech. These values were used to guide early method optimization work for the quantitative LC/MS/MS based proteomics method described previously. Strong correlation ($r^2 \geq 0.83$, $p <$ 0.0001) was obtained for the midazolam 1'-hyrdoxylation activities provided by the

manufacturer when compared to both the LC/MS/MS protein content and the Western blot values, as shown in Figure 10. Strong correlation ($r^2 \geq 0.78$, $p < 0.0001$) was also obtained for the testosterone 6β-hydroxylation activities provided by the manufacturer when compared to the LC/MS/MS protein content and the Western blot values, as shown in Figure 11.

Figure 10. Correlation of CYP3A protein content with midazolam 1'-OH activity (XenoTech)

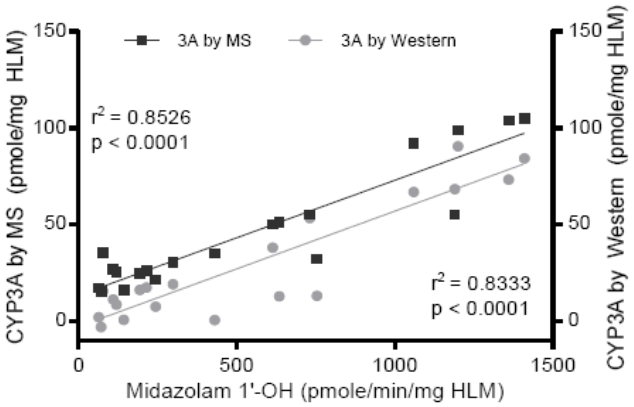
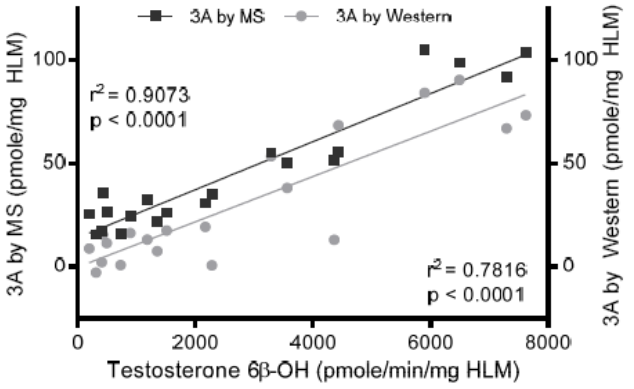


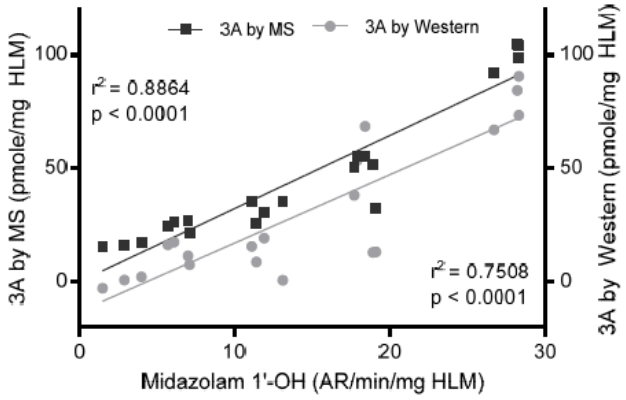
Figure 11. Correlation of CYP3A protein content with testosterone 6β-OH activity (XenoTech)



Caution should be used in regarding the manufacturer-provided values as an accurate representation of the samples used during experimentation. Typically, the characterization work is performed by the manufacturer immediately following isolation of the microsomal fraction and is not necessarily representative of the sample by the time it is used at the bench. A wide variety of factors can affect the viability of enzymatic preparations in the time that is likely to

elapse between their characterization and use. These include but are not limited to the duration of time since isolation and exposure to multiple freeze/thaw cycles or other forms of temperature variation. Therefore, confirmation of CYP3A activity was obtained by repeating the midazolam 1'-hydroxylation assay. Strong correlation ($r^2 \geq 0.75$, $p < 0.0001$) was observed with the CYP3A protein contents calculated by LC/MS/MS and Western blot analysis, as shown in Figure 12.

Figure 12. Correlation of CYP3A protein content and midazolam 1'-OH activity (in-house)



4.3.2 Chemical Inhibition of DB289 / DB1230 Metabolite Formation

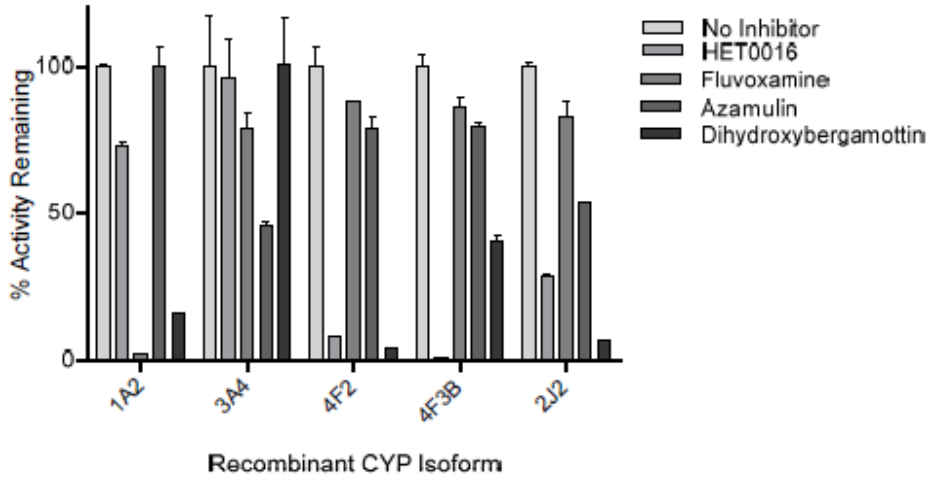
The lack of any significant correlation between CYP4F protein content and either DB289 or DB1230 metabolite formation is likely influenced by a lack of substrate specificity. The coherence between the LC/MS/MS protein content and the immunoquantified values suggests that the lack of correlation is likely not due to incorrectly calculated protein content. Even if the absolute values of protein content were inaccurate, the relative trend should demonstrate some degree of correlation to the rate of DB289 and DB1230 metabolite formation if CYP4F2 and CYP4F3B were the only enzymes catalyzing this reaction. Based on the previous work by Wang et al (2006), it is not unexpected that the potential for alternative pathways of primary metabolite formation exists. Several additional recombinant CYP enzymes known to be expressed in the liver (CYP1A2, CYP3A4, CYP2J2) were implicated in DB289 primary metabolite formation,

though these were thought to have a minor contribution to metabolite formation in more complex matrices such as liver microsomes. Preliminary evaluation of the individual donor HLM samples for CYP2J2 content (data not shown) suggested that the samples being tested contained low levels of CYP2J2 (< 1.7 pmole/mg HLM). Additionally, lack of significant inhibition (< 20%) of primary metabolite formation was observed in previous studies in HLM samples by 1 μM ebastine, which was shown to inhibit recombinant CYP2J2-mediated DB289 metabolism by greater than 95% (Wang et al., 2006). Therefore, no additional experiments were performed with CYP2J2 inhibitors during the subsequent chemical inhibition studies.

To further investigate the potential for alternate routes of metabolism of DB289 and DB1230, chemical inhibition experiments were performed using an arachidonic acid CYP-mediated metabolism inhibitor (HET0016), a CYP1A2 inhibitor (fluvoxamine), and several CYP3A inhibitors (azamulin and DHB). Prior to incubation with HLMs, the inhibitors were incubated with recombinant CYP enzymes and either DB289 or DB1230 to evaluate the specificity of enzyme inhibition. With respect to DB289, it was determined that HET0016 was a potent inhibitor of CYP4F2 (92% inhibition), CYP4F3B (99% inhibition), and CYP2J2 (71% inhibition). HET0016 did not significantly inhibit metabolism of DB289 by CYP1A2 (27% inhibition) or CYP3A4 (4% inhibition). Fluvoxamine was a potent inhibitor of CYP1A2-mediated DB289 metabolism (98% inhibition) and did not significantly inhibit DB289 metabolism by CYP3A4 (21% inhibition), CYP4F2 (12% inhibition), CYP4F3B (24% inhibition), or CYP2J2 (27% inhibition). Azamulin was observed to be a moderate inhibitor of DB289 metabolism by CYP3A4 (54% inhibition) and CYP2J2 (46% inhibition), but did not significantly inhibit CYP1A2 (no inhibition), CYP4F2 (21% inhibition), or CYP4F3B (20% inhibition). DHB was observed to be a potent inhibitor of DB289 metabolism by CYP4F2 (95% inhibition), CYP1A2 (84% inhibition), and CYP2J2 (93% inhibition). Significant inhibition of

DB289 metabolism by CYP4F3B (59% inhibition) was observed by DHB. Interestingly, as a CYP3A inhibitor, DHB did not inhibit CYP3A4-mediated DB289 metabolism. However, control incubations using midazolam as the substrate confirmed that the concentration of DHB was appropriate, as potent inhibition (93% inhibition) of midazolam 1'-OH formation was observed (data not shown). Significant DB289 metabolite formation was observed in the no inhibitor control for all recombinant enzymes studied. These results are summarized in Figure 13.

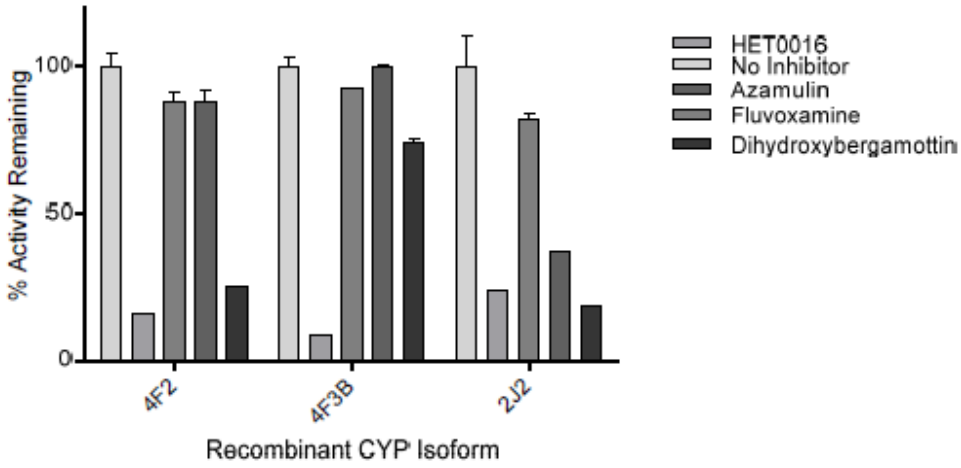
Figure 13. Chemical inhibition of DB289 metabolism by CYP-selective inhibitors



When performing the same control experiments with DB1230, it was observed that this substrate demonstrated an improved specificity towards the CYP4F enzymes. Specifically, negligible DB1230 metabolite formation was observed following incubation with either CYP1A2 or CYP3A4. In this context, negligible implies that the "no inhibitor" control incubations contained near identical amounts of DB1230 primary metabolite as the "no NADPH" control incubations, suggesting that the initial DB1230 substrate solution contained trace levels of the primary metabolite prior to incubation with the various enzymes. However, significant metabolism of DB1230 was observed by CYP4F2, CYP4F3B, and CYP2J2 and the

full panel of inhibitors was evaluated for specificity with respect to these enzymes. With respect to DB1230, it was determined that HET0016 was a potent inhibitor of CYP4F2 (84% inhibition), CYP4F3B (91% inhibition), and CYP2J2 (76% inhibition). Fluvoxamine did not significantly inhibit DB1230 metabolism by CYP4F2 (12% inhibition), CYP4F3B (7% inhibition), or CYP2J2 (18% inhibition). Azamulin was observed to be a potent inhibitor of DB1230 metabolism by CYP2J2 (63% inhibition), but did not significantly inhibit CYP4F2 (12% inhibition) or CYP4F3B (0% inhibition). DHB was observed to be a potent inhibitor of DB1230 metabolism by CYP4F2 (75% inhibition) and CYP2J2 (81% inhibition). Moderate inhibition of DB1230 metabolism by CYP4F3B (26% inhibition) was observed by DHB. These results are summarized in Figure 14.

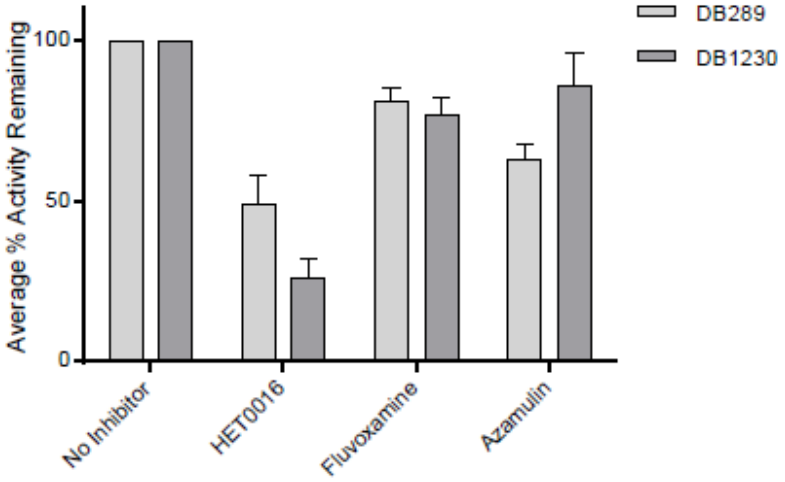
Figure 14. Chemical inhibition of DB1230 metabolism by CYP-selective inhibitors



Based on the observed specificity of the control experiments, the inhibitors HET0016, fluvoxamine, and azamulin were selected for incubation with the panel of individual donor HLM samples and either DB289 or DB1230. An additional double inhibition experiment was performed with DB289 which included incubation with fluvoxamine and azamulin in an attempt to reduce the contribution from non-CYP4F metabolic pathways. Significant inhibition of primary metabolite formation was observed in the presence of HET0016 for both DB289 and

DB1230. The average (range) percent activity remaining in the panel of individual donor HLM samples following incubation with 0.1 µM HET0016 was observed to be 49% (34 - 66%) and 26% (17 - 41%) for DB289 and DB1230, respectively. Inhibition of primary metabolite formation was also observed for fluvoxamine and azamulin, though the magnitude of inhibition was significantly less than that observed for HET0016. The average (range) percent activity remaining for individual donor HLM samples following incubation with 3 µM fluvoxamine was observed to be 81% (73 - 88%) and 77% (68 - 86%) for DB289 and DB1230, respectively. The average (range) percent activity remaining for individual donor HLM samples following incubation with 3 µM azamulin was observed to be 63% (53% - 74%) and 86% (71% - 108%) for DB289 and DB1230, respectively. The average (range) percent activity remaining for the individual donor HLM samples following the double incubation experiment with 3 µM fluvoxamine and 3 µM azamulin was observed to be 58% (49% - 70%) for DB289. These results are summarized in Figure 15.

Figure 15. Chemical inhibition of DB289 / DB1230 metabolism by HLM



The extensive inhibition of primary metabolite formation by HET0016 supports the hypothesis that CYP4F enzymes play a major role in the biotransformation of DB289 and DB1230. However, significantly more activity remained in the current study (~49%) following

incubation with 0.1 μM HET0016 than was reported previously (~22%) using pooled HLM prepared from 50 donor livers (Wang et al., 2006). Additionally, some inhibition of metabolite formation (by 19-23%) was observed by fluvoxamine for both DB289 and DB1230 in the current study whereas it had previously been reported that fluvoxamine did not inhibit metabolite formation even though recombinant CYP1A2 was shown to be very active towards the metabolism of DB289.

The residual activities for DB289 metabolite formation in the presence of fluvoxamine and fluvoxamine/azamulin were plotted against the CYP4F protein content determined by LC/MS/MS to evaluate if inhibiting alternate pathways of metabolism improved the correlation with CYP4F protein content. Additionally, the residual activity for DB1230 metabolite formation in the presence of azamulin was plotted against the CYP4F protein content determined by LC/MS/MS. As fluvoxamine had minimal effect on DB1230 metabolic activity, the double inhibition experiment was not performed for this substrate. No correlation was observed for DB289 metabolite formation in the presence of both fluvoxamine (r^2=0.01, p = 0.69) and fluvoxamine/azamulin (r^2=0.004, p = 0.79), as shown in Figure 16. No correlation was observed for DB1230 metabolite formation in the presence of azamulin (r^2=0.09, p = 0.21), as shown in Figure 17.

Figure 16. Residual DB289 metabolite formation by HLM in presence of chemical inhibitors

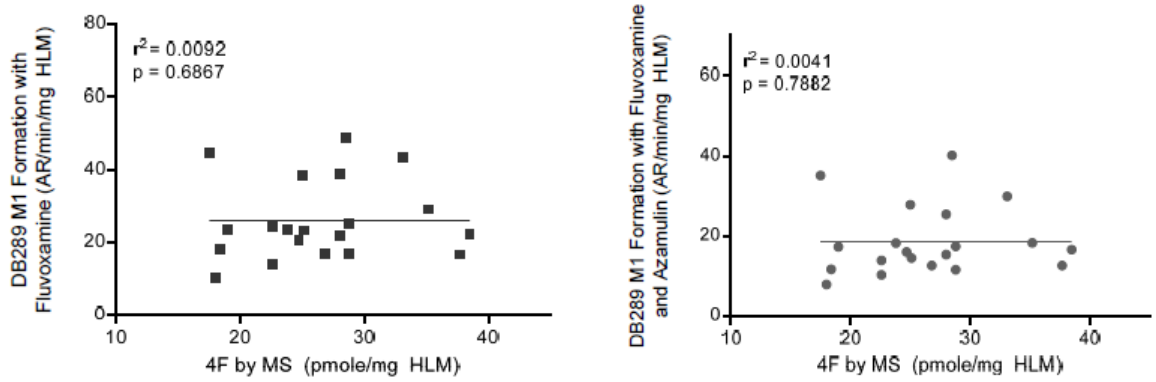
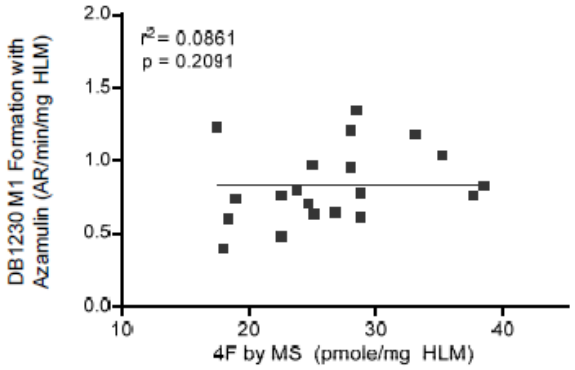


Figure 17. Residual DB1230 metabolite formation by HLM in presence of azamulin



The lack of an improvement in correlation following incubation with the various chemical inhibitors suggests that the poor correlation of CYP4F protein content and primary metabolite formation for DB289 and DB1230 is not solely the result of alternative metabolic pathways. This hypothesis is supported by the fact that poor correlation was also observed for DB1230, which was shown through the recombinant control experiments to be less susceptible to metabolism by other hepatic CYP enzymes.

4.4 Summary

The correlation of protein content and enzyme activity was evaluated for CYP4F and CYP3A enzymes using multiple probe substrates. No correlation was observed between the CYP4F protein content and DB289 / DB1230 primary metabolite formation, though it has been shown previously that CYP4F2 and CYP4F3B are the major hepatic and enteric enzymes responsible for this biotransformation (Wang et al., 2006). The coherence between the LC/MS/MS and Western blot enzyme protein content suggest that the lack of correlation is likely due to complicating factors involved in the metabolic activity assay. Chemical inhibition assays suggested that while CYP4F enzymes are responsible for a significant portion of the observed primary metabolite formation for these substrates, additional pathways for primary metabolite formation are likely present. The reduction in metabolite formation upon inclusion of selective

CYP1A2 and CYP3A4 inhibitors suggests that these enzymes may play a role in metabolite formation; however, the extent of this effect needs to be better characterized before firm conclusions can be drawn.

Strong correlation ($r^2 \geq 0.7480$) was observed for all CYP3A marker activities. This is not unexpected as CYP3A continues to be one of the most studied subfamily of CYPs. As such, the metabolic pathways that are used for evaluation of CYP3A enzyme activity have been well characterized over decades of use. This work only highlights the limitations of predicting enzyme expression based on enzyme activity for lesser known enzymes, such as the CYP4F subfamily. Substrate specificity is critical if one is to follow a particular biotransformation and attempt to relate the observed activity to an estimate of protein expression (Alterman et al., 2005). Additionally, factors such as auto-inhibition and auto-induction of the enzyme system under consideration by a probe substrate can further confound interpretation of metabolite formation data. However, even with these limitations, use of probe substrates and marker activities to evaluate enzyme expression remains an important part of any drug development toolkit.

One potential explanation for the lack of correlation between CYP4F protein content and the observed primary metabolite formation for DB289 and DB1230 may involve altered catalytic activity due to genetic polymorphisms known to exist in CYP4F2. Two functional variants have been recently identified, specifically the W12G and V433M variants (Bardowell et al., 2010). The V433M variant has been implicated in inter-individual variability associated with warfarin through its effects on Vitamin K1 metabolism (McDonald et al., 2009) and both variants have been implicated in altered catalytic activity of Vitamin E. Interestingly, the variants have been linked to both increased and decreased catalytic activity and the effects have also been observed to be substrate dependent. Additionally, it has been suggested that the variants can affect

metabolic activity through reduction of the amount of enzyme present (by inhibiting translation or inducing degradation) or by attenuating the catalytic activity of the enzyme itself. The latter may help explain the poor correlation observed between CYP4F protein content and primary metabolite formation for DB289 and DB1230, though additional studies are required to confirm this hypothesis.

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