THE EFFECT OF CIMETIDINE ON
DEXTROMETHORPHAN O-DEMETHYLASE ACTIVITY
OF HUMAN LIVER MICROSOMES AND RECOMBINANT CYP2D6

By

MARIA MADEIRA

B.Sc. (Honours, Toxicology), University of Guelph, 2000

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
Faculty of Pharmaceutical Sciences
Division of Pharmacology and Toxicology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
March 2003

© Maria Madeira, 2003

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Faculty of Pharmaceutical Sciences

The University of British Columbia
Vancouver, Canada

Date April 8, 2003

ABSTRACT

Cimetidine is a histamine H₂-receptor antagonist used in the treatment of peptic ulcer disorders. There have been numerous clinical observations of cimetidine drug-drug interactions, in some cases involving known substrates of cytochrome P450 (P450) 2D6. One explanation for these observations is impaired hepatic metabolism via inhibition of P450s, the primary mediators of hepatic phase I metabolism of drugs. Cimetidine has been demonstrated to inhibit various P450 enzymes *in vitro*, including CYP2D6, but the inhibition constants are generally higher than the plasma concentrations measured following *in vivo* administration. In rats, the *in vitro* selectivity and potency of cimetidine can be increased by preincubating the inhibitor and NADPH with hepatic microsomes, and the formation of a metabolite-intermediate complex has been implicated. The effect of preincubation on inhibition of CYP2D6 by cimetidine has not been investigated. The objective of this project was to further characterize the effect of cimetidine on CYP2D6 *in vitro* using human liver microsomes and recombinant CYP2D6.

Dextromethorphan O-demethylase activity was used as a probe of CYP2D6 activity. Metabolite formation was quantified using a high performance liquid chromatography assay with fluorescence detection. The $V_{\max}$ and K_m of this reaction were 0.82 ± 0.06 nmol/min/nmol P450 and 4.1 ± 0.1 μ M, respectively, in microsomes; and 15.9 ± 0.8 nmol/min/nmol P450 and 1.4 ± 0.6 μ M, respectively, with recombinant CYP2D6. With human liver microsomes, cimetidine was a competitive inhibitor of CYP2D6 with a K_i of 38.0 ± 5.3 μ M; the observed inhibition was not effected by preincubation. With the recombinant enzyme, cimetidine acted as a mixed inhibitor with a K_i of 102.5 ± 16.8 μ M. Preincubation with the inhibitor and NADPH led to a further decrease in activity of 11% to 18% in a manner that was consistent with mechanism-based inactivation. The effect of preincubation was attenuated by including quinidine in the preincubation reaction. The k_{inact} and K_I were estimated to be 0.03 min^{-1} and 19.2 μ M, respectively, while the $t_{1/2}$ of inactivation was 25 minutes.

In the present study, cimetidine acted as an inhibitor of dextromethorphan O-demethylation in both *in vitro* systems. With recombinant CYP2D6, loss of activity was enhanced with preincubation. In both cases, the effect of cimetidine as an inhibitor of CYP2D6 was modest and does not fully explain the clinical findings.

TABLE OF CONTENTS

	Page
Abstract	ii
Table of contents	iii
List of tables	vi
List of figures	viii
List of abbreviations	xii
1. Introduction	1
1.1 Cytochromes P450	2
1.2 Cytochrome P450 2D6	5
1.2.1 Pharmacogenetics of CYP2D6	5
1.2.2 CYP2D6*10	7
1.3 <i>In vitro</i> methods used to study drug metabolism	8
1.3.1 Human liver microsomes and recombinant enzymes	8
1.3.2 Use of probe substrates	9
1.3.3 Dextromethorphan <i>O</i> -demethylase activity of CYP2D6	10
1.4 Inhibition of cytochrome P450 enzymes	13
1.4.1 Competitive, non-competitive, and mixed inhibition	13
1.4.2 Reversible inhibition	14
1.4.2.1 Quinidine	15
1.4.3 Quasi-irreversible and irreversible inhibition	15

1.4.3.1 Triacetyloleandomycin and 1-aminobenzotriazole	17
1.4.4 Clinical consequences of P450 inhibition	17
1.5 Cimetidine	18
1.5.1 Drug-drug interactions with cimetidine	20
1.5.2 Inhibition of CYP2D6 by cimetidine	21
1.6 Hypothesis and Objectives	22
2. Materials and methods	25
2.1 Chemicals	25
2.2 Enzyme incubations	25
2.2.1 Incubation conditions for human liver microsomes	25
2.2.2 Incubation conditions for recombinant CYP2D6	26
2.2.3 Preincubation experiments	27
2.3 HPLC assay methodology	28
2.3.1 Assay conditions	28
2.3.2 Calibration curves	29
2.3.3 Assay validations	29
2.4 Data analysis	30
3. Results	32
3.1 Assay validation	32
3.2 Optimization of incubation conditions	43
3.3 Studies with human liver microsomes	49
3.3.1 Kinetic constants of dextromethorphan O-demethylation	49
3.3.2 Effect of quinidine and cimetidine on dextromethorphan O-demethylase activity	52

3.3.3	Preincubation studies with cimetidine	57
3.3.4	Mechanism-based inhibition with triacetyloleandomycin and 1-aminobenzotriazole	70
3.3.5	Summary of studies with human liver microsomes	70
3.4	Studies with recombinant CYP2D6	73
3.4.1	Kinetic constants of dextromethorphan <i>O</i> -demethylation	73
3.4.2	Effect of quinidine and cimetidine on dextromethorphan <i>O</i> -demethylase activity	73
3.4.3	Preincubation studies with cimetidine	79
3.4.4	Preincubation of recombinant CYP2D6 with 1-aminobenzotriazole and quinidine	85
3.4.5	Effect of preincubation of the K_i of inhibition by cimetidine	88
3.4.6	Effect of quinidine on inactivation of recombinant CYP2D6 by cimetidine	95
3.4.7	Determination of k_{inact} of cimetidine	97
3.4.8	Summary of studies with recombinant CYP2D6	97
4.	Discussion	99
5.	Future studies	111
5.1	Studies with human liver microsomes	111
5.2	Studies with recombinant CYP2D6	112
5.3	Studies with recombinant CYP2D6*10	114
6.	Summary & Conclusions	115
7.	References	117

LIST OF TABLES

	page
Table 1: Intra-assay precision and accuracy of quality control (QC) samples prepared with human liver microsomal matrix.	39
Table 2: Inter-assay precision and accuracy of quality control (QC) samples prepared with human liver microsomal protein matrix.	40
Table 3: Intra-assay precision and accuracy of quality control (QC) samples prepared with inactivated recombinant human CYP2D6.	41
Table 4: Inter-assay precision and accuracy of quality control (QC) samples prepared with inactivated recombinant human CYP2D6.	42
Table 5: Summary of the kinetic constants for dextromethorphan O-demethylation by pooled human liver microsomes determined in three separate experiments.	51
Table 6: Summary of estimated K_i values for inhibition of dextromethorphan O-demethylation by quinidine and cimetidine with pooled human liver microsomes, determined in three separate experiments.	56
Table 7: Preincubation of human liver microsomes with cimetidine (25 μ M) for up to 30 minutes.	58
Table 8: Effect of adding NADPH to the preincubation mixture every 60 minutes versus only once at the beginning of the preincubation reaction.	61
Table 9: Summary of the kinetic constants for dextromethorphan O-demethylation by recombinant CYP2D6 determined in three separate experiments.	75

Table 10:	Summary of estimated K_i values for inhibition of dextromethorphan <i>O</i> -demethylation by quinidine and cimetidine with recombinant CYP2D6, determined in three separate experiments.	78
Table 11:	Summary of K_i values for the inhibition of dextromethorphan <i>O</i> -demethylase activity of recombinant CYP2D6 by cimetidine.	91

LIST OF FIGURES

	Page
Figure 1: The P450 catalytic cycle	4
Figure 2: Metabolic pathways of dextromethorphan demethylation	10
Figure 3: Chemical structure of cimetidine and its major metabolites	19
Figure 4: Chromatographic separation of pure standards of dextromethorphan and its metabolites.	34
Figure 5A: Representative chromatogram of dextrorphan and 3-methoxymorphinan produced in a microsomal incubation.	35
Figure 5B: Representative chromatogram of dextrorphan and 3-methoxymorphinan produced in an incubation with recombinant CYP2D6.	36
Figure 6: Representative calibration curves for (A) dextrorphan (DOR), (B) 3-hydroxymorphinan (3-HM), and (C) 3-methoxymorphinan (3-MM).	37
Figure 7: Optimization of microsomal protein concentration	44
Figure 8: Optimization of incubation time with pooled human liver microsomes.	45
Figure 9: Optimization of NADPH concentration with human liver microsomes.	46
Figure 10: Optimization of recombinant CYP2D6 concentration.	47
Figure 11: Optimization of incubation time with recombinant CYP2D6.	47

Figure 12:	Optimization of NADPH concentration with recombinant CYP2D6.	48
Figure 13:	Rate of formation of dextrorphan by human liver microsomes.	50
Figure 14:	Inhibition of dextromethorphan <i>O</i> -demethylase activity in human liver microsomes by quinidine.	53
Figure 15:	Effect of cimetidine free base and hydrochloride salt on dextromethorphan <i>O</i> -demethylase activity in human liver microsomes.	54
Figure 16:	Inhibition of dextromethorphan <i>O</i> -demethylase activity in human liver microsomes by cimetidine.	55
Figure 17:	Effect of addition of NADPH to the preincubation mixture every 30 minutes.	60
Figure 18:	Preincubation of pooled human liver microsomes with cimetidine for up to 120 minutes.	63
Figure 19:	Preincubation of human liver microsomes from individual HG 95 with cimetidine for up to 120 minutes.	66
Figure 20:	Preincubation of human liver microsomes from individual HG 64 with cimetidine for up to 120 minutes.	67
Figure 21:	Effect of preincubation of human liver microsomes with cimetidine for 90 minutes, followed by incubation with 50 μ M dextromethorphan, on dextromethorphan <i>O</i> -demethylase activity in human liver microsomes.	68
Figure 22:	Effect of preincubation of human liver microsomes with cimetidine for 90 minutes, followed by incubation with 50 μ M or 300 μ M dextromethorphan, on dextromethorphan <i>N</i> -demethylase activity of human liver microsomes.	69
Figure 23:	Inactivation of dextromethorphan <i>N</i> -demethylase activity in human liver microsomes by triacetyloleandomycin (TAO).	71

Figure 24:	Inactivation of dextromethorphan O-demethylase activity in human liver microsomes by 1-aminobenzotriazole (ABT).	72
Figure 25:	Rate of formation of dextrorphan by recombinant CYP2D6.	74
Figure 26:	Inhibition of dextromethorphan O-demethylase activity of recombinant CYP2D6 by quinidine.	76
Figure 27:	Inhibition of dextromethorphan O-demethylase activity of recombinant CYP2D6 by cimetidine.	77
Figure 28:	Preincubation of recombinant CYP2D6 with cimetidine for 5, 10, and 20 minutes, followed by incubation with 1 μ M dextromethorphan.	80
Figure 29:	Preincubation of CYP2D6 with cimetidine (50 μ M) for 20 minutes, followed by incubation with 1 μ M dextromethorphan.	81
Figure 30:	Preincubation of recombinant CYP2D6 with cimetidine for 5, 10, and 20 minutes, followed by incubation with a saturating concentration of dextromethorphan.	83
Figure 31:	Preincubation of CYP2D6 with cimetidine for 20 minutes, followed by incubation with a saturating concentration of dextromethorphan.	84
Figure 32:	Mechanism-based inactivation of recombinant CYP2D6 by 1-aminobenzotriazole (ABT).	86
Figure 33:	Lack of mechanism-based inactivation of recombinant CYP2D6 by quinidine.	87
Figure 34:	Inhibition of recombinant CYP2D6 by cimetidine following 20 minutes preincubation using the dilution method.	89
Figure 35:	Dixon plot for the inhibition of recombinant CYP2D6 by cimetidine following 20 minutes preincubation.	90

Figure 36A:	Dixon plot for the inhibition of recombinant CYP2D6 by cimetidine following 20 minutes preincubation using saturating substrate concentrations.	93
Figure 36B:	Lineweaver-Burk transformation of the data presented in figure 33A.	94
Figure 37:	Effect of quinidine on inactivation of recombinant CYP2D6 by cimetidine.	96
Figure 38:	Inactivation of recombinant CYP2D6 by cimetidine.	98

LIST OF ABBREVIATIONS

μL	microlitre
μM	micromolar
ABT	1-aminobenzotriazole
AUC	area under the plasma concentration versus time curve
$^{\circ}\text{C}$	degrees Celsius
Cl_{int}	<i>in vitro</i> intrinsic clearance, equivalent to $V_{\text{max}}/K_{\text{m}}$
CV	coefficient of variation
CYP2D6	cytochrome P450 2D6
dex	dextromethorphan
DOR	dextrorphan
EM	extensive metabolizer
3-HM	3-hydroxymorphinan
HPLC	high performance liquid chromatography
K_{i}	inhibition constant; concentration of inhibitor required for half-maximal inhibition
K_{I}	concentration of inactivator required for half-maximal inactivation
k_{inact}	rate constant for inactivation
K_{m}	Michaelis-Menten constant; concentration of substrate required to achieve half-maximal activity
LOQ	limit of quantitation
mg	milligram
min	minute
mL	millilitre
mM	millimolar

3-MM	3-methoxymorphinan
NADPH	β -nicotinamide adenine dinucleotide phosphate
nM	nanomolar
nmol	nanomole
P450	cytochrome P450
PM	poor metabolizer
pmol	picomole
PVDF	polyvinylidene fluoride
QC	quality control
t_{1/2}	half-life
TAO	triacetyloleandomycin
V_{max}	maximal velocity of reaction

Chapter 1

INTRODUCTION

The effects of drug therapy can vary markedly among individuals and one of the major factors contributing to this inter-individual variation is altered capacity of the enzymes involved in drug metabolism. Two of the ways in which enzyme activity can be altered are the expression of polymorphic forms of the enzyme, or by drug-drug interactions resulting in enzyme inhibition. Variant alleles can arise from a number of mutational events and can result in increased, diminished, or absent enzyme activity. Such polymorphisms are known to exist for over 20 phase I and phase II drug metabolizing enzymes, including several cytochrome P450 (P450) enzymes (Ingelman-Sundberg 1999). P450s, which are the predominant mediators of phase I metabolism of xenobiotics, are also susceptible to inhibition by a large number of drugs. Therefore, there is a potential for a compound effect caused by the inhibition of polymorphically expressed enzymes, which may result in unexpected responses.

Cimetidine is an example of a drug that has been implicated in a number of clinical interactions, some of which are thought to involve CYP2D6. CYP2D6 is one of the most extensively characterized polymorphic P450s and over 75 alleles, conferring varying degrees of metabolic activity, have been identified (Ingelman-Sundberg and Evans 2001). The frequency of a given allele can vary greatly between ethnic populations, as is seen with the *CYP2D6*10* allele, which is almost absent in Caucasians (Marez et al. 1997), but has a frequency of over 50% in some Asian populations (Garcia-Barcelo et al. 2000). Expression of the *CYP2D6*10* protein results in an enzyme with decreased activity. It is possible that the effect of cimetidine as an inhibitor of CYP2D6 may be dependent on the phenotype

of the expressed enzyme, and this could explain some of the clinical observations that have previously not had a clear mechanism.

In vitro models can be used to predict where drug-drug interactions are likely to occur, and an individual's genotype for polymorphically expressed drug metabolizing enzymes, such as CYP2D6, can be accurately determined. Information regarding how an inhibitor affects the activity of an enzyme, or any of its genetic variants, could therefore be used to determine where adjustments in dosing are needed to reduce the potential for toxicity in individuals with altered metabolic capacity.

1.1 Cytochromes P450

P450s are heme-thiolate proteins that catalyze mono-oxygenase reactions in the presence of molecular oxygen, NADPH, and the accessory enzyme NADPH-cytochrome P450 reductase (Gonzalez 1989). P450s are expressed in all eukaryotes and some prokaryotes; however, the expression of specific enzymes is highly species-specific and, in some cases, sex- and age-dependent (Omura 1999). Individual enzymes are divided into families (denoted by arabic numerals) and subfamilies (denoted by letters) based on the similarity of their amino acid sequences. Enzymes within the same family demonstrate less than 40% homology with proteins in other families, while enzymes within the same subfamily share at least 59% homology in their amino acid sequences (Gonzalez and Nebert 1990). Individual genes are distinguished by a second arabic numeral.

In humans, at least 14 different P450 families exist (Nelson *et al.* 1996). Families 1 through 3 are predominantly involved in the metabolism of xenobiotics, while the other families catalyze endogenous reactions, such as those involved in the synthesis of steroid hormones, leukotrienes, prostaglandins and bile acids (Smith *et al.* 1998). P450s differ from most enzymes in that they have broad and overlapping substrate specificity. As a result of this, a large number of structurally diverse chemicals can be metabolized and several enzymes may be involved in the metabolism of a single substrate. In humans, P450s are responsible for the majority of phase I reactions involved in the

metabolism of drugs and other xenobiotics. This step functions to render generally hydrophobic compounds more susceptible to phase II conjugation, which results in the addition of a hydrophilic moiety to the compound. The end result is the formation of a more water-soluble species which can be readily excreted.

The majority of the xenobiotic metabolizing P450s are expressed in the liver, but can also be found in extrahepatic tissues such as the gastrointestinal tract, kidneys, and lungs (Smith *et al.* 1998). At a subcellular level, these P450s are embedded within the membrane of the endoplasmic reticulum. During the isolation of these enzymes from cells, the endoplasmic reticular membrane is disrupted and forms small vesicles called microsomes, which contain the P450 enzymes.

The catalytic cycle of P450s can be generally described by 8 steps (Guengerich 2001), which are depicted in figure 1. The substrate binds to the enzyme in step one, while the iron is in the ferric state. In step 2, the iron is reduced by the transfer of a single electron from NADPH-P450 reductase. Molecular oxygen then binds to the ferrous iron (step 3), resulting in an $\text{Fe}^{2+}\cdot\text{O}_2$ complex. A second electron is then transferred (step 4), followed by the addition of a proton (step 5). The nature of the iron-oxygen complex in these, and subsequent, steps is unclear. In step 6, the O-O bond is cleaved and, by addition of another proton, water is released. The product is then formed (step 7) and released from the active site of the enzyme (step 8).

Figure 1: The P450 catalytic cycle. See text for a description of each step. Adapted from Guengerich (2001).

1.2 Cytochrome P450 2D6

Only a single enzyme in the CYP2D subfamily has been detected in the human liver: CYP2D6 (Nelson *et al.* 1996). The human CYP2D6 enzyme was first purified by Distlerath *et al.* (1985) and was later cloned by Kimura *et al.* (1989). Located on the long arm of chromosome 22, the *CYP2D* gene locus contains three genes: *CYP2D6*, which codes for the functional enzyme, and *CYP2D7P* and *CYP2D8P*. The latter two genes are not transcribed; *CYP2D7P* contains an insertion mutation which disrupts the reading frame, while *CYP2D8P* contains multiple nucleotide deletions and insertions (Kimura *et al.* 1989). Although CYP2D6 represents only 2% of total hepatic CYP content (Shimada *et al.* 1994), it plays a major role in drug metabolism and is involved in the metabolism of over 50 clinically important drugs (Hasler *et al.* 1999). CYP2D6 substrates, which include various antiarrhythmics, β -blockers, antihypertensives, antidepressants, neuroleptics, and opioid derivatives (Cholerton *et al.* 1992), have little in common other than the presence of a nitrogen which can be protonated at physiological pH and is thought to form an ion pair with the negatively charged aspartate residue located in position 301 of the CYP2D6 amino acid sequence (Ellis *et al.* 1995). This nitrogen atom is usually found 5 to 7 Å from the site of hydroxylation (Lewis *et al.* 1999).

1.2.1 Pharmacogenetics of CYP2D6

It has been recognized for over 30 years that genetic factors play a major role in inter-individual differences in response to drug therapy (Kalow 1968, 1971). Genetic polymorphisms of the genes encoding drug transporters, receptors, or metabolizing enzymes can lead to the expression of proteins with altered activity towards drugs. Much research has been focused on characterizing and determining the functional consequences of mutations within the genes of drug metabolizing enzymes. These variant alleles can arise from a number of mutational events including gene deletions, single nucleotide polymorphisms (which can in turn result in frameshift, missense, nonsense, or splice-site mutations), or gene duplications (Ingelman-Sundberg *et al.* 1999) and can result in absent, decreased, or

increased enzyme activity or expression. Clinically, polymorphisms can have several effects on the outcome of pharmacotherapy, including therapeutic failure or an increased susceptibility to toxicity. These effects are generally most noticeable with drugs that are frequently prescribed or have a narrow therapeutic index (Bronsen and Gram 1989). The frequency of a polymorphic allele can vary greatly between ethnic populations so that the responses of different ethnic groups to the same drug may be quite different. As a result of this, dosages of drugs primarily metabolized by polymorphic enzymes may have to be determined for individual populations to reduce the negative influence of these polymorphisms on effective therapy (Tanaka 1999).

The CYP2D6 polymorphism was first identified by Mahgoub *et al.* (1977). Using debrisoquine (a drug which is predominantly metabolized by CYP2D6 [Dalen *et al.* 1998]) as a probe, metabolic ratios were determined by measuring the amount of the parent drug relative to the metabolite 4-hydroxydebrisoquine in the subjects' urine. Two groups of individuals, classified as non-metabolizers and extensive metabolizers, were identified based on the bimodal distribution of the calculated metabolic ratios and the non-metabolizer phenotype was found to be associated with the inheritance of a single autosomal recessive gene. Further studies, however, have shown that the classification of CYP2D6 phenotypes is more complex and four phenotypic groups are now recognized: poor, intermediate, extensive, and ultrarapid metabolizers, with extensive metabolizers (EMs) having "normal" enzyme activity and poor metabolizers (PMs) having no CYP2D6 activity (Van der Weide and Steijns 1999). Certain alleles are associated with specific phenotypic groups. However, the divisions between these groups are not well defined and the large number of possible combinations of CYP2D6 alleles adds an additional complexity to the issue. Over 75 variant alleles, in addition to the 'wild-type' allele (denoted *CYP2D6*1* [Marez *et al.* 1997]), have been identified and the functional effects of all of these polymorphisms have not been determined (Ingelman-Sundberg and Evans 2001). Most of these alleles, however, are rare and an individual's phenotype can be accurately predicted by screening for a small group of alleles known to be prevalent in a given population. For example, in the

German population, screening for four genes provided an accurate assessment of an individual's phenotype (Sachse *et al.* 1998).

1.2.2 CYP2D6*10

Of the many CYP2D6 variants that have been identified, CYP2D6*10, which results in an intermediate metabolizer phenotype, is of particular importance due to its prevalence in Asian populations. The occurrence of the poor metabolizer phenotype in Asians is rare. However, impaired metabolism of CYP2D6 substrates was observed in studies with Chinese patients being treated with various neuroleptics agents, compared to Caucasians being treated with the same drugs (Potkin *et al.* 1984, Rudorfer *et al.* 1984). Further research lead to the identification of the CYP2D6*10 allele, which is characterized by the presence of three nucleotide substitutions: C¹⁸⁸→T, G¹⁷⁴⁹→C, and G⁴²⁶⁸→C (Marez *et al.* 1997). Several other forms of the allele have been identified, which differ based on the presence of additional nucleotide substitutions, but code for enzymes that display the same catalytic activity (Marez *et al.* 1997).

Estimates of the CYP2D6*10 allele frequency range from 0.41 to 0.68 in Japanese populations, where this variant represents the most common CYP2D6 allele (Tateishi *et al.* 1999, Kubota *et al.* 2000), and 0.64 in the Chinese population (Garcia-Barcelo *et al.* 2000). The proportion of homozygous carriers of this allele has been estimated to be from 0.17 among Japanese (Tateishi *et al.* 1999) to 0.45 and 0.41 in Korean and Chinese populations, respectively (Yoon *et al.* 1997, Garcia-Barcelo *et al.* 2000). In contrast, the frequency of the CYP2D6*10 allele in Caucasians is less than 0.02 (Sachse *et al.* 1997, Grise *et al.* 1998). Various clinical studies have demonstrated that individuals who have the CYP2D6*10 allele experience a reduced ability to metabolize CYP2D6 substrates (Wang *et al.* 1993, Fukada *et al.* 1999, Yoon *et al.* 2000) which is directly related to the number of CYP2D6*10 alleles present (Johansson *et al.* 1994). This reduction in clearance is due to two factors: a decreased expression of the enzyme and a reduced affinity of the mutant enzyme for substrates. Johansson *et al.*

(1994) demonstrated that the presence of the C¹⁸⁸→T mutation, which results in a Pro→Ser amino acid substitution at position 34 of the amino acid sequence, leads to the expression of an unstable gene product, thus resulting in lower enzyme levels. In studies of the catalytic activity of CYP2D6*10, Fukada *et al.* (2000) found that while the V_{max} is unaffected, the K_m of both bufuralol 1'-hydroxylation and venlafaxine O-demethylation is increased by 5- to 10-fold compared to CYP2D6*1.

1.3 *In vitro* methods used to study drug metabolism

1.3.1 *Human liver microsomes and recombinant enzymes*

There are a number of different *in vitro* models available for the study of drug metabolism, which include cultured hepatocytes, liver tissue slices, subcellular fractions such as microsomes, and heterologously expressed drug metabolizing enzymes (Rodrigues 1994). The most commonly used models in studies of cytochrome P450-related drug metabolism are hepatic microsomes and heterologously expressed enzymes. These two *in vitro* systems each have their own advantages and disadvantages.

Microsomes offer the advantage of having a full complement of P450s, with each enzyme present in ratios and concentrations typically seen *in vivo* (Crespi 1995). Levels of enzyme expression and activity can vary greatly between individuals, however, and the source of human microsomes used can influence experimental results (Crespi and Penman 1997). This influence can be minimized by using pooled microsomes from a number of donors, which has the effect of averaging out any extreme differences in enzyme expression. An additional advantage found with microsomes is that the enzymes are present in their "natural" environment in terms of the lipid membrane in which they are embedded and the number of accessory proteins present, such as NADPH-cytochrome P450 reductase. Changes in either of these factors can lead to altered enzyme activity (Venkatakrishnan *et al.* 2000). Some of the disadvantages associated with microsomes are the absence of phase II metabolizing enzymes, which results in an incomplete representation of metabolic pathways seen *in vivo*, and the fact that inhibitory

antibodies or chemicals (both of which are rarely specific for an single enzyme) are usually required to study the activity of a single enzyme (Crespi 1995).

The study of single enzymes is facilitated by the use of heterologously expressed, or recombinant, P450s. Expression systems that are commonly used include bacteria, yeast, insect, and human cells (Crespi and Miller 1999). Experiments carried out using this type of model system can provide qualitative information regarding the enzymes involved in the metabolism of drugs, but give no indication as to how much an individual enzyme contributes to metabolism *in vivo* (Crespi and Penman 1997). As a result of this, it is difficult to relate data derived from recombinant P450s to what might be seen *in vivo*. A number of different methods of extrapolating this data from recombinant to microsomal models have been developed, including the use of relative activity scaling factors (Nakajima *et al.* 1999, Shimada *et al.* 1999, Venkatakrishnan *et al.* 2000).

1.3.2 Use of probe substrates

The activity of P450 enzymes can be studied using probe substrates: compounds that are metabolized to a known product by the enzyme of interest. Preferably, only a single enzyme is involved in the production of the metabolite; with P450s, however, this is rarely the case. Due to their overlapping substrate specificity, several enzymes may be involved in the metabolism of a single compound, and even in the production of a single metabolite. However, if the different enzymes exhibit sufficiently different affinities for the substrate, the specificity of the reactions may be increased under defined experimental conditions. There are several experimental approaches used to determine which P450s are involved in the metabolism of a compound, which include correlating the enzyme activity of interest to that of a known marker activity of a specific P450 enzyme; assessing the effect of chemical inhibitors or inhibitory antibodies; or by using individual recombinant enzymes to determine which P450s are capable of catalyzing the reaction (Halpert *et al.* 1994). Often, a combination of these methods is used to provide a more accurate estimate of the specificity of the reaction.

1.3.3 Dextromethorphan O-demethylase activity of CYP2D6

Dextromethorphan is a commonly used *in vivo* and *in vitro* probe of CYP2D6 activity. The metabolic pathways of dextromethorphan have been well characterized in both human liver microsomes (Dayer *et al.* 1989, von Moltke *et al.* 1998) and with recombinant P450 enzymes (Yu and Haining 2001), and its use as an *in vivo* marker has been demonstrated (Capon *et al.* 1996, Jones *et al.* 1996). Dextromethorphan undergoes O- or N-demethylation to form dextrorphan or 3-methoxymorphinan, respectively, and these two metabolites are both further demethylated to form the secondary metabolite, 3-hydroxymorphinan (figure 2).

Figure 2: Metabolic pathways of dextromethorphan demethylation. The major P450 enzyme responsible for the formation of each metabolite is shown in bold lettering

The formation of dextrorphan, the O-demethylated metabolite, has been well documented *in vitro* and the conditions under which this pathway is specific for CYP2D6 activity are known. Studies with human liver microsomes have shown the formation of dextrorphan to be biphasic, with the high affinity pathway having a K_m in the range of 2.3 to 5.3 μM and the low affinity pathway having a K_m approximately ten-fold higher (Dayer *et al.* 1989, Kerry *et al.* 1994), suggesting that at least two different enzymes contribute to the formation of this metabolite. While this pattern is typically seen in microsomes from extensive metabolizers of debrisoquine, one-site kinetics are observed with microsomes from poor metabolizers, with a K_m similar to that of the low affinity pathway in extensive metabolizers (Kerry *et al.* 1994). The absence of a high affinity enzyme pathway in poor metabolizers, who demonstrate little or no CYP2D6 activity, suggests that this pathway is primarily mediated by CYP2D6. The effect of selective inhibitors of P450 enzymes on dextromethorphan O-demethylase activity was investigated by Schmider *et al.* (1997) and confirms the role of CYP2D6 as the high affinity enzyme involved in dextrorphan formation. Quinidine, a CYP2D6 inhibitor, reduced the formation of dextrorphan by 80%, while other CYP2D6 substrates, such as bufuralol, debrisoquine, and sparteine competitively inhibited dextromethorphan O-demethylation in microsomes from extensive metabolizers with K_i values which ranged from 7.5 μM for bufuralol to 45 μM for sparteine, when the concentrations of dextromethorphan used were well below the k_m for the low affinity pathway (Dayer *et al.* 1989). In contrast, inhibitors of CYPs 1A1/2 (α -naphthaflavone), 2C9 (sulfaphenazole), and 3A (triacytyleandomycin and ketoconazole) had no effect (Dayer *et al.* 1989). *In vivo* administration of quinidine has been shown to inhibit dextrorphan formation in extensive metabolizers, but has no effect in poor metabolizers (Capon *et al.* 1996).

Studies with individual P450 enzymes (von Moltke *et al.* 1998, Yu and Haining 2001) have shown that recombinant CYPs 2D6, 3A4, 2C9, 2C19, and 2B6 are all capable of metabolizing dextromethorphan to dextrorphan, but CYP2D6 has the highest intrinsic clearance (Cl_{int} , which is represented by the ratio of V_{max}/K_m for the reaction). Using *in vitro* relative activity factor scaling to

determine the relative contribution of each enzyme, it was estimated that at substrate concentrations up to two-fold higher than the K_m for the high affinity pathway, CYP2D6 is responsible for approximately 70% of dextrophan formation, while at higher substrate concentrations, the involvement of CYP2C9 increases (von Moltke *et al.* 1998).

The *N*-demethylation of dextromethorphan has been proposed as a probe of CYP3A activity, although its suitability is questionable. Using recombinant P450 enzymes, Yu and Haining (2001) showed that the formation of 3-methoxymorphinan was mediated by CYPs 2B6, 3A4, 2C19, 2D6, 2C9, and 2C19. CYP2B6 and 3A4 demonstrated the highest intrinsic clearance, but the overall contribution of CYP2B6 in human liver microsomes or *in vivo* was predicted to be minor due to the low hepatic expression of this enzyme, and CYP3A4 was estimated to be responsible for greater than 90% of dextromethorphan *N*-demethylation activity. In a similar study, however, CYP3A4 was predicted to be responsible for only 50% of *N*-demethylation activity, with CYPs 2C9 and 2C19 equally responsible for the residual activity (von Moltke *et al.* 1998). This second estimate is in agreement with the results from several studies with human liver microsomes, where maximal inhibition of dextromethorphan *N*-demethylase activity by triacetyloleandomycin and indinavar, both of which were shown to completely inhibit recombinant CYP3A4/5 activity, was only 60% with human liver microsomes (Wang and Unadkat 1999). Similarly, anti-CYP3A antibodies resulted in an inhibition of 40-50% (Gorski *et al.* 1994, Schmider *et al.* 1997).

The formation of the secondary metabolite, 3-hydroxymorphinan, is a result of the *N*-demethylation of dextrophan or the *O*-demethylation of 3-methoxymorphinan. The *O*-demethylation pathway, mediated by CYP2D6, is thought to be the major route of formation. The formation of 3-hydroxymorphinan by the *N*-demethylation pathway is mediated by CYP3A4 (Yu and Haining 2001).

1.4 Inhibition of cytochrome P450 enzymes

A large number of compounds, including many drugs, are known to inhibit P450 enzymes. Impairment of any of the steps of the P450 catalytic cycle can lead to loss of enzyme activity; however, the steps involving the binding of the substrate, binding of molecular oxygen, and oxidation of the substrate (steps 1, 3, and 7, respectively, in figure 1) are most susceptible (Ortiz de Montellano and Correia 1995). The resulting inhibition may be relatively selective, and affect only one or two closely related P450 enzymes, or may be more general, and depends on several factors, including the chemical properties of the inhibitor and its mechanism of action (Lin and Lu 1998).

There are two systems of classification used to categorize enzyme inhibitors. The first system describes inhibitors based on their effect on the kinetic parameters of the catalyzed reaction and includes competitive, non-competitive, and mixed inhibitors. The second system addresses the mechanism by which the inhibitor acts. In this case, inhibitors can be classified as reversible, quasi-irreversible, or irreversible. The latter method of classification is often more useful when discussing inhibitors of P450s because it is more specific (for example, non-competitive inhibition can be caused by both reversible and irreversible inhibitors).

1.4.1 *Competitive, non-competitive, and mixed inhibition*

Competitive inhibitors act by binding to the active site of the enzyme, thus preventing the substrate from binding and being metabolized. They are also often, but not always, capable of acting as substrates for the enzymes they inhibit. Competitive inhibitors are characterized by reducing the apparent affinity (or increasing the K_m , the concentration of substrate required to reach half-maximal activity) of the enzyme for its substrate, but have no effect on the maximal rate of the reaction (V_{max}). Increasing concentrations of the substrate will displace the inhibitor from the active site and reverse its effect on enzyme activity. Non-competitive inhibitors, on the other hand, bind to a site distinct from the substrate binding site. As a result, these inhibitors do not affect the enzyme's affinity for the

substrate. Inhibition cannot be overcome by increasing the concentration of the substrate and the $V_{\max}$ of the enzyme will be decreased. Mixed inhibitors act by a combination of competitive and non-competitive inhibition and can affect both the K_m and the $V_{\max}$ (Copeland 1996, Lin and Lu 1998).

1.4.2 Reversible inhibition

Reversible inhibitors can bind to P450s by one of two modes: substrate (or productive) binding and ligand binding (Testa 1990). Inhibition by the first mode occurs when two compounds compete for binding to the active site of the same enzyme. Maximal inhibition is seen when one substrate binds with a greater affinity, but has a much lower catalytic turnover, than the other (Ortiz de Montellano and Correia 1995). Enzyme activity is restored when the inhibitor is metabolized or diffuses away from the active site (Murray 1987). Ligand binding usually involves nitrogen-containing compounds, such as quinolones, imidazoles, and pyridines. The lone pair of electrons on the nitrogen forms a strong coordinate bond with the ferric form of the prosthetic heme, resulting in a type II difference spectrum with a Soret band in the region of 420-425 nm (Halpert 1995). Inhibition due to ligand binding prevents substrate turnover by increasing the redox potential of the enzyme, thus making the electron transfer step more difficult, and by occupying the binding site required for molecular oxygen (Ortiz de Montellano and Correia 1995).

Compounds rarely form only one type of bond, and those which are able to bind to both the heme iron and the protein moiety through lipophilic interactions are inherently more potent (Ortiz de Montellano and Correia 1995). The selectivity of these inhibitors is determined by how tightly they fit into the active site of the enzyme, and whether the inhibitor's orientation in the active site allows for the formation of a coordinate bond with the ferric heme (Halpert 1995). The relative potency of reversible inhibitors is defined by the inhibition constant (K_i) which is a measure of the inhibitor's binding capacity for the enzyme (Copeland 1996).

1.4.2.1 Quinidine

Quinidine, an example of a reversible P450 inhibitor, is a potent and selective inhibitor of CYP2D6 *in vitro*. Studies with human liver microsomes have shown that quinidine is a competitive inhibitor of dextromethorphan O-demethylase activity with a K_i ranging from 0.028 to 0.4 μM (Kobayashi *et al.* 1989, Ching *et al.* 1995, Bourri *et al.* 1996). Furthermore, concentrations of quinidine which result in more than 80% inhibition of CYP2D6 activity inhibit other P450 enzymes less than 10% (Newton *et al.* 1995). The potency of quinidine as an inhibitor is related to its ability to bind to both the substrate-binding site and to co-ordinate with heme iron (Ortiz de Montellano and Correia 1995). The binding of quinidine to rat hepatic microsomes results in a type II binding spectrum, suggesting that the inhibitor is interacting with the ferric iron (Murray 1984). Unlike the majority of reversible, competitive inhibitors, quinidine does not undergo metabolic biotransformation by the enzyme it inhibits; instead, its metabolism is primarily mediated by CYP3A (Guengerich *et al.* 1986).

1.4.3 Quasi-irreversible and irreversible inhibition

These types of inhibitors require catalytic activation to a reactive species by the target enzyme; for this reason, they are also referred to as being “mechanism-based”. In this thesis, inhibition of enzyme activity by a mechanism-based process will be referred to as “inactivation” to distinguish it from loss of activity due to reversible inhibition. There are three steps involved in mechanism-based inactivation: the inactivator reversibly binds to the active site of the enzyme, undergoes catalytic conversion, and the reactive species binds to the enzyme leading to loss of activity (Halpert *et al.* 1994). Quasi-irreversible and irreversible inactivators differ in the way in which they bind to the target enzyme. With quasi-irreversible inactivators, a metabolite intermediate (MI) complex is formed, in which the product of P450-dependent metabolism binds tightly, but not covalently, to the heme group (Ortiz de Montellano and Correia 1995). These inactivators are described as quasi-irreversible because although the MI complex can be dissociated under certain conditions *in vitro*, such as incubation with a

highly lipophilic compound or oxidation of the heme group with potassium ferricyanide, these conditions are rarely achieved *in vivo* and enzyme activity is only restored on synthesis of new protein. Irreversible inactivators, on the other hand, lead to loss of enzyme activity through covalent modification of the enzyme. This can occur by several mechanisms, including binding to the prosthetic heme group of the P450 enzyme, the apoprotein moiety, or through activation of the heme group to products that subsequently covalently bind to the apoprotein (Halpert 1995). Mechanism-based inactivators are generally more selective and potent than reversible inhibitors because the compound must be able to bind to the active site of a specific P450 enzyme, must have the properties required of a substrate in order to be catalytically activated, and then alter the enzyme structure, resulting in its permanent removal from the catalytic pool (Ortiz de Montellano and Correia 1995).

Several criteria must be met for an inactivator to be classified as mechanism-based (Silverman 1995). Inactivation is time-dependent, as it is limited by the rate of conversion of the inactivator to the reactive species, and is saturable, with maximal inactivation seen when a sufficient concentration of the inhibitor is present to occupy all of the enzyme molecules. The presence of a substrate of the target enzyme will slow the rate of inactivation by restricting access of the inactivator to the active site, through a process called substrate protection. Enzyme inactivation is irreversible, and follows a 1:1 stoichiometry: one inhibitor molecule will inactivate one enzyme molecule. Finally, enzyme inactivation must occur prior to the release of the reactive species from the active site of the enzyme.

While reversible inhibitors are defined by their K_i value, mechanism-based inactivation is more appropriately described by the rate constant for inactivation (k_{inact}) and the concentration of inactivator required to achieve a half-maximal rate of inactivation (K_I). The k_{inact} value is a complex expression which combines the rate constants that define binding of the inhibitor, its catalytic conversion to a reactive species, and the rate at which this reactive species is either released from the active site as a product or binds to and inactivates the enzyme (Silverman 1995).

1.4.3.1 Triacetyloleandomycin and 1-aminobenzotriazole

Triacetyloleandomycin (TAO) and 1-aminobenzotriazole (ABT) are two compounds which act as mechanism-based inactivators of P450s. TAO is a highly selective inactivator of CYP3A (Chang *et al.* 1994) which acts by the formation of a metabolite-intermediate complex with the ferrous heme of the enzyme, a complex which can be readily dissociated with potassium ferricyanide (Pessayre *et al.* 1982, Lindstrom *et al.* 1993). The parent compound is first *N*-dealkylated to a primary amine, which then undergoes further metabolism to a nitroso intermediate. This species then coordinates the heme iron through the lone pair of electrons on the nitroso functional group (Ortiz de Montellano and Correia 1995). Unlike TAO, ABT causes irreversible inactivation of P450 enzymes through alkylation of the heme group by a reactive species, which has been demonstrated through the isolation of abnormal porphyrin products following incubation of hepatic microsomes with ABT (Ortiz de Montellano and Mathews 1981). The destruction of microsomal P450s can result in a loss of almost 80% of total P450 following administration of ABT to rats, and is both time- and concentration-dependent (Colby *et al.* 1995). In comparison to TAO, the selectivity of ABT for specific P450 enzymes is not as great. At lower concentrations of the inactivator, both human recombinant CYPs 3A and 2D6 are inactivated with IC₅₀ values less than 10 μ M, while CYPs 1A2, 2B6, 2C9, and 2C19 are all inactivated to a lesser extent, with IC₅₀ values between 50 and 250 μ M (Balani *et al.* 2002).

1.4.4 Clinical consequences of P450 inhibition

Drug-drug interactions can occur when one drug inhibits the hepatic clearance of another. Impairment of the activity of drug-metabolizing enzymes can have several clinical consequences, including an exaggerated pharmacological effect or increased incidence of adverse effects due to elevated level of the parent drug, or therapeutic failure in the case of prodrugs that require activation by metabolic enzymes (Benetti and Bani 1998). Enzyme inhibition is most likely to have clinical consequences when the affected drug has a low therapeutic index and small changes in plasma

concentrations can lead to significant alteration in the toxicity profile of the drug. The duration and severity of the effect is dependent on the type of inhibition and the degree to which the inhibited enzyme is involved in the metabolism of concurrently administered drugs. Interactions due to competitive inhibition last only as long as the inhibitor is present in the liver at sufficient concentrations; enzyme activity is restored as the inhibitor gradually diffuses away from the active site. With mechanism-based inactivation, however, recovery is dependent on the synthesis of new enzyme and therefore requires a longer period of time (Murray 1997).

Inhibition of the P450 enzymes involved in the metabolism of a drug will lead to a decrease in the drug's clearance, with the magnitude of the effect related to the role the inhibited enzyme plays in the overall clearance of the drug. Changes in clearance are reflected by changes in the area under the plasma concentration time curve (AUC): a decrease in clearance will result in an increased AUC (Lin and Lu 1998). The pharmacological response or toxicity of a drug can also be related to the AUC (Ito *et al.* 1998), therefore this pharmacokinetic parameter provides a good assessment of the effect of *in vivo* enzyme inhibition.

1.5 Cimetidine

Cimetidine is an H₂-receptor antagonist used in the treatment of gastric acid disorders (Feldman and Burton 1990). Structurally, cimetidine is a substituted imidazole (figure 3), which is slightly basic.

The pharmacokinetic properties of cimetidine have been well documented. Following oral administration, the drug is rapidly absorbed, with a maximal plasma concentration of 6 to 9 μ M achieved 60 to 90 minutes after administration of a 300 mg dose (Brogden *et al.* 1978, Somogyi and Gugler 1983). Plasma concentrations are proportional to dose when the drug is administered in single doses of up to 800 mg (Bodemar *et al.* 1981). The major route of elimination of cimetidine is renal excretion. Within 24 hours of dosing, 50 to 80% of the drug is recovered unchanged in the urine

(Somogyi and Gugler 1983). The remainder of the drug is metabolized to four products: cimetidine-N'-glucuronide, cimetidine sulfoxide, hydroxymethyl cimetidine, and guanylurea cimetidine (Somogyi and Gugler 1983). Severe adverse effects are rarely seen with cimetidine treatment, however, the drug has been implicated in a number of drug-drug interactions.

Figure 3: Chemical structure of cimetidine and its major metabolites

1.5.1 Drug-drug interactions with cimetidine

The first study of a drug interaction with cimetidine was reported by Serlin *et al.* in 1979, where concurrent treatment with warfarin was found to potentiate the effect of the anticoagulant. This effect was associated with an increased plasma concentration and decreased clearance of warfarin. Since this initial report, interactions with over 40 drugs have been reported (reviewed in Somogyi and Gugler 1982, Feldman and Burton 1990), including benzodiazepines, theophylline, phenytoin, and propranolol. Cimetidine's ability to impair the clearance of a wide variety of drugs led to the initial assumption that it was a general inhibitor of P450s (Puurunen *et al.* 1980, Pelkonen and Puurunen 1980). This was supported by early *in vitro* studies where it was demonstrated that cimetidine was able to reversibly bind to the ferric iron of rat microsomal P450, resulting in a typical type II binding spectrum, and two different binding affinities were detected, indicating that at least two separate forms of P450s were interacting with cimetidine (Rendic *et al.* 1979). Further *in vivo* and *in vitro* studies were conducted, which addressed specific P450 activities, and it was found that some enzymes are refractory to inhibition by cimetidine. In rats, *in vivo* administration of cimetidine inhibited activities specific to CYPs 2C11 and 2C6, but not CYPs 2A1, 2B1/2, or 3A1/2 (Chang *et al.* 1992A, Levine *et al.* 1998). When the same enzyme activities were examined *in vitro*, cimetidine was a weak inhibitor of CYPs 2B1/2 and 3A1/2 in addition to CYP2C11; however, when rat hepatic microsomes were preincubated with cimetidine and NADPH, the potency of inhibition of CYP2C11 approximated that seen *in vivo* (Chang *et al.* 1992B). At high concentrations, cimetidine appears to act as a weak, general P450 inhibitor; following preincubation both the potency and specificity of inhibition is increased, suggesting that cimetidine may be metabolized to an inhibitory species. Spectral evidence has shown that a metabolite-intermediate complex is formed when cimetidine is incubated with rat hepatic microsomes and NADPH, and the formation of this complex can be blocked by anti-rat CYP2C11 antibodies (Levine and Bellward 1995). Preincubation of cimetidine with human liver microsomes and NADPH also resulted in an inhibition of dapsone metabolism, with increasing inhibition seen with longer

preincubation times (Tingle *et al.* 1991). Mechanism-based inactivation of P450 enzymes by cimetidine is further supported by a delay in the onset of the cimetidine-drug interaction. For example, with propranolol, clearance was reduced by 33% after one day of treatment with cimetidine; following five days, however, a 51% decrease was observed (Reiman *et al.* 1981).

1.5.2 Inhibition of CYP2D6 by cimetidine

Based on clinical and *in vitro* data, CYP2D6 appears to be one of the P450 enzymes inhibited by cimetidine in humans. The clearance of several CYP2D6 substrates *in vivo* is decreased by approximately 30% to 50% by cimetidine, including propranolol (Reimann *et al.* 1981), imipramine (Miller and Macklin 1983, Abernethy *et al.* 1984), desipramine (Amsterdam *et al.* 1984), and sparteine (Schellens *et al.* 1989). However, with other CYP2D6 substrates, such as nortriptyline, the effect of cimetidine is inconsistent. While cimetidine was found to increase plasma concentrations of nortriptyline in one reported case (Miller *et al.* 1983), a subsequent controlled clinical trial involving six subjects failed to observe the same effect (Henauer *et al.* 1984). Similar results were observed with desipramine, where the AUC of this drug was increased in some, but not all subjects (Amsterdam *et al.* 1984, Steiner and Spina 1987); however, this effect was explained by the individuals' CYP2D6 phenotype. In the EMs, the AUC of desipramine was increased by an average of 50%, whereas PMs, who do not express functional CYP2D6, demonstrated no change in the AUC. CYP2D6 phenotype, therefore, appears to play a significant role in determining the effect of cimetidine *in vivo*. Although this effect is not large enough to effectively convert the EMs to a PM phenotype (Philip *et al.* 1989), the lack of effect in PMs provides strong evidence that cimetidine is an effective inhibitor of CYP2D6 *in vivo*.

In vitro inhibition studies have determined the K_i of inhibition by cimetidine on several CYP2D6 substrates. Bufuralol hydroxylase activity was competitively inhibited with a K_i of 50 to 55 μM (Knodell *et al.* 1991, Furuta *et al.* 2001), while dextromethorphan O-demethylation was inhibited with an IC_{50} of 200 μM when a substrate concentration of 25 μM was used (Martinez *et al.* 1999).

Compared to maximum plasma concentrations of approximately 10 μM , however, these values are too high to fully explain the clinical observations. The effect of preincubation on inhibition of CYP2D6 by cimetidine has not been investigated.

1.6 Hypothesis and objectives

The *in vivo* and *in vitro* studies described above have shown that cimetidine is a P450 inhibitor. *In vitro* treatment with a high concentration of cimetidine results in general inhibition of P450 enzymes; however, cimetidine is a more selective and more potent inhibitor at the lower concentrations seen *in vivo*. The effect seen after *in vivo* administration of cimetidine can be emulated *in vitro* when hepatic microsomes are preincubated with the inhibitor, as was demonstrated with inhibition of rat CYP2C11, which leads to the formation of a metabolite-intermediate complex.

In humans, cimetidine has been shown to decrease the clearance of many therapeutically important drugs, some of which are known substrates of CYP2D6. Inhibition of CYP2D6 by cimetidine has also been observed *in vitro*, but the estimated K_i values are approximately 50 μM , which is above the maximum plasma concentration of approximately 10 μM attained during therapy. Clinical observation has also shown that drug interactions with CYP2D6 substrates are not consistent among individuals. The CYP2D6 enzyme displays a large degree of genotypic and phenotypic variation, which impacts the metabolism of CYP2D6 substrates, and which may explain these observations.

Although both *in vitro* and clinical data indicate that CYP2D6 is inhibited by cimetidine, the mechanism of this inhibition is unclear. Based on the effect of cimetidine on rat CYP2C11 and the discrepancy between the *in vitro* inhibitory potency relative to plasma levels of cimetidine, it is possible that mechanism-based inhibition may be occurring. The inhibitory effect of cimetidine *in vivo* is also dependent on CYP2D6 phenotype. One allelic variant of this enzyme, CYP2D6*10, has been shown to be less susceptible to *in vitro* competitive inhibition by compounds such as sparteine, debrisoquine, and norfluoxetine, compared to CYP2D6*1 (Ramamoorthy *et al.* 2001). **Therefore, it could be**

hypothesized that the CYP2D6*10 variant is less susceptible to inhibition by cimetidine than CYP2D6*1. Were this found to be the case, this may provide an explanation for the inconsistency in clinically observed interactions.

In order to begin testing this hypothesis, the first premise to be addressed is the mechanism by which cimetidine acts as an inhibitor of CYP2D6. **The specific hypothesis tested in this thesis is: cimetidine is a mechanism-based inactivator of CYP2D6.**

The specific objectives of this project were:

1. to develop and validate a quantitative assay for the measurement of the metabolites of dextromethorphan;
2. using human liver microsomes, to determine the effect of quinidine and cimetidine on dextromethorphan O-demethylase activity, with and without preincubation;
3. using recombinant CYP2D6, to determine the effect of quinidine and cimetidine on dextromethorphan O-demethylase activity, with and without preincubation.

If cimetidine is acting as a mechanism-based inactivator of CYP2D6, preincubation of the enzyme with cimetidine should lead to an increase in the potency of the inhibitor, as seen by a decrease in the estimated K_i value. Quinidine will be utilized to show that an increase in inhibition with preincubation is not observed with reversible inhibitors. As a competitive inhibitor of CYP2D6, quinidine's effect should be similar both with and without preincubation. Finally, two different *in vitro* model systems, human liver microsomes and recombinant CYP2D6, will be used. If a metabolite of cimetidine is involved in the inhibition of CYP2D6, this metabolite may be formed by a P450 enzyme other than CYP2D6. A lack of effect with preincubation with the individual recombinant enzyme could have various possible explanations: cimetidine may not be acting as a mechanism-based inactivator or, alternatively, another P450 enzyme could be involved in the production of the reactive

species. Therefore, studies will initially be done with microsomes, in which all hepatic P450 enzymes are present.

Chapter 2

MATERIALS & METHODS

2.1 Chemicals

Dextromethorphan hydrobromide, cimetidine, quinidine, 1-aminobenzotriazole (ABT), and triacetyloleandomycin (TAO) were purchased from Sigma Chemical Co. (St. Louis, MO). Dextrorphan (DOR) and 3-methoxymorphinan hydrochloride (3-MM) were obtained from Gentest Corp. (Woburn, MA), while 3-hydroxymorphinan (3-HM) was from RBI (Oakville, ON). Also purchased from Sigma Chemical Co. were β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and reagent grade magnesium chloride. HPLC grade potassium phosphate monobasic and methanol, and perchloric acid were bought from Fisher Scientific (Toronto, ON). Reagent grade potassium phosphate monobasic and dibasic were from Fisher Scientific and Anachemica (Montreal, PQ), respectively.

2.2 Enzyme incubations

2.2.1 Incubation conditions for human liver microsomes

Pooled human liver microsomes and microsomes from two individual donors with high CYP2D6 activity (catalog numbers HG 64 and HG 95) were purchased from Gentest Corp. (Woburn

MA). The protein and P450 concentration for each lot of microsomes was provided by the manufacturer.

Preliminary experiments were performed to ensure that the protein concentration and incubation time used were within the linear range of metabolite formation, and that the concentration of NADPH was in excess (see section 3.2).

The incubation mixtures consisted of microsomal protein (0.5 mg/mL), dextromethorphan, 50 mM potassium phosphate (pH 7.4), and 1 mM magnesium chloride. Reaction tubes were placed in a 37 °C shaking water bath for 2 minutes to allow for temperature equilibration prior to initiation of the reaction with 1 mM NADPH dissolved in 50 mM potassium phosphate (pH 7.4). For inhibition studies, cimetidine or quinidine was added to the incubation mixture before initiation of the reaction with NADPH. All reactions were carried out in a final volume of 200 µL in glass tubes. Reactions were terminated after 20 minutes by precipitation of the protein with 20 µL ice-cold perchloric acid and mixing with a vortex. Samples were then centrifuged at 3000 x *g* for 10 minutes in a Beckman model J-6B centrifuge. The supernatant was removed with a 1 mL syringe, passed through a 0.22 µm PVDF membrane syringe driven filtration unit (Millipore Ltd., Bedford MA), and transferred to autosampler vials fitted with 150 µL conical inserts (both from VWR, Edmonton AB) for HPLC analysis.

Solutions of dextromethorphan, cimetidine, and quinidine were made in 50 mM potassium phosphate (pH 7.4), with minimal amounts of methanol used to aid in solubility. In all cases, the final volume of methanol in the microsomal incubation was less than 0.1% of the total incubation volume, which is below the concentration found to inhibit CYP activity (Busby *et al.* 1998).

2.2.2 Incubation conditions for recombinant CYP2D6

Baculovirus-insect-cell-expressed human CYP2D6 supersomes (recombinant CYP2D6) and control supersomes, which consisted of microsomes produced from the expression system transfected with the wild-type vector, were purchased from Gentest Corp. The protein and P450 concentrations

were provided by the manufacturer. As with the human liver microsomes, preliminary experiments were conducted to determine the optimal incubation conditions with respect to CYP2D6 and NADPH concentration, and incubation time (see section 3.2). No formation of dextrophan was observed when control microsomes were incubated with dextromethorphan and NADPH.

Incubation mixtures consisted of 2.5 pmol P450/mL, dextromethorphan, 100 mM potassium phosphate (pH 7.4), and 3.3 mM magnesium chloride. For inhibition studies, cimetidine or quinidine was added to the incubation mixture. The incubation mixtures were preincubated for 2 minutes at 37 °C in a shaking water bath before initiation of the reaction with 1 mM NADPH. All reactions were carried out in a final volume of 200 µL in glass tubes. After 15 minutes, reactions were terminated with 20 µL ice-cold perchloric acid. The samples were then centrifuged and filtered, as described for the human liver microsome samples, prior to analysis by HPLC.

2.2.3 Preincubation experiments

The following procedure was used for the majority of the preincubation experiments; any variations in this method are noted in the figure legends where appropriate. For experiments with human liver microsomes, the preincubation mixture consisted of microsomal protein (2 mg/mL), inhibitor, 50 mM potassium phosphate (pH 7.4), and 1 mM magnesium chloride. Where the recombinant enzyme was used, the preincubation mixture contained CYP2D6 (10 pmol/mL), inhibitor, 100 mM potassium phosphate (pH 7.4), and 3.3 mM magnesium chloride.

The preincubation mixture was warmed in a 37 °C shaking water bath for 2 minutes before the reaction was initiated by the addition of 1 mM NADPH. At various time points following the start of the reaction, 50 µL aliquots were removed from the preincubation mixture and added to reaction tubes containing dextromethorphan, 1 mM NADPH, potassium phosphate, and magnesium chloride in a volume of 150 µL. The reactions were then incubated for a further 20 or 15 minutes, for samples containing human liver microsomes or recombinant CYP2D6, respectively, and were terminated with

20 μ L perchloric acid. In the final incubation step, the concentration of microsomal protein was 0.5 mg/mL, and the concentration of recombinant CYP2D6 was 2.5 pmol/mL.

Control preincubation reactions, in which microsomal protein (2 mg/mL) or recombinant CYP2D6 (10 pmol/mL) were preincubated with 1 mM NADPH in the absence of inhibitor, were assayed at the same time. The activity in the inhibitor-treated groups was compared to the control activity at each time point.

2.3 HPLC Assay Methodology

2.3.1 Assay conditions

The chromatographic system consisted of a 1525 binary pump, 717plus autosampler, and 474 scanning fluorescence detector (all from Waters, Milliford MA). Operation of all equipment, as well as data acquisition and peak integration, was managed with Waters' Breeze software. Separation was achieved on a Waters SymmetryShield RP₁₈ column (4.6 mm x 150 mm, 5 μ m particle size) equipped with a SymmetryShield RP₁₈ Sentry guard column (3.9 mm x 20 mm, 5 μ m particle size). A mobile phase of 60% 50 mM potassium phosphate (adjusted to pH 4.75 with 50 mM potassium phosphate dibasic) and 40% methanol was pumped through the column at 1 mL/minute at ambient temperature. All mobile phase components were HPLC grade, including the water used to prepare the 50 mM potassium phosphate, which was produced on site by reverse osmosis and subsequent purification using a Milli-Q filtration system (Millipore Ltd., Bedford MA). The mobile phase was degassed by vacuum filtration through 0.45 μ m filters (Millipore Ltd.) prior to use. An injection volume of 25 μ L was used and the fluorescence excitation and emission wavelengths were set at 280 nm and 310 nm, respectively.

2.3.2 Calibration curves

Stock solutions of the metabolite standards (200 μM) were prepared in distilled water and stored in 1 mL aliquots at -20°C in glass screw-top vials (VWR Scientific, Edmonton AB). Working solutions of 20, 2, and 0.2 μM were prepared on the day of each experiment by dilution of freshly-thawed 200 μM stock solutions in potassium phosphate (50 or 100 mM, for microsomal or recombinant CYP2D6, respectively). Standard ($n=3$) and quality control (QC) samples ($n=5$) were prepared using a method which paralleled the incubation conditions. Mixtures containing either microsomal protein (0.5 mg/mL) and 50 mM potassium phosphate, or recombinant CYP2D6 (2.5 pmol/mL) and 100 mM potassium phosphate, were incubated for 2 minutes at 37°C in a shaking water bath. The protein was subsequently precipitated by the addition of 20 μL perchloric acid, and the samples were vortex mixed and placed on ice for 10 minutes. Appropriate volumes of the working solutions were then added to give metabolite concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2.5, and 5 μM , in a volume of 200 μL . The samples were then centrifuged at $3000 \times g$ for 10 minutes, and the supernatant was filtered through a Millex-GV syringe driven filter unit with a 0.22 μm PVDF membrane (Millipore Ltd.) prior to analysis by HPLC. Binding of the analytes to the filter membrane was less than 5%. Calibration curves for each of the three metabolites were constructed by plotting the concentration of the standard against the integrated peak area. The equations of the curves were determined by unweighted linear regression using Microsoft Excel (1995).

2.3.3 Assay Validation

The inter- and intra-assay accuracy and precision were evaluated by determining the percent bias (% bias) and percent coefficient of variation (% CV), respectively, of the high, mid, and low QC samples. Inter-assay variability was assessed by analyzing a set of standards ($n=3$) and QC samples ($n=5$) on five separate days, while the intra-assay variability was assessed by analyzing a set of standards

(n=3) and QC samples (n=5) in a single run. QC samples were quantitated by back calculation of the concentration using the equation of the calibration curve. The % bias and % CV were calculated using the following equations:

$$\% \text{ bias} = \frac{\text{mean concentration of 5 QC samples} - \text{nominal concentration}}{\text{nominal concentration}} \times 100\%$$

$$\% \text{ CV} = \frac{\text{standard deviation of 5 QC samples}}{\text{mean concentration}} \times 100\%$$

2.4 Data Analysis

All kinetic modeling was done with the SigmaPlot enzyme kinetics module, version 1.1 (SPSS Inc., Chicago IL, 2001). The K_m and V_{max} values were determined using the Michaelis-Menten equation, where V represents the measured enzyme velocity at substrate concentration S :

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

K_i values were estimated using the following equations:

for competitive inhibition:
$$v = \frac{V_{max}}{(1 + (K_m/S)(1 + I/K_i))}$$

for noncompetitive inhibition:
$$v = \frac{V_{max}}{((1 + I/K_i)(1 + K_m/S))}$$

for mixed inhibition:
$$v = \frac{V_{max}}{\frac{((K_m/S)(1 + I/K_i))}{1 + I/(\alpha/K_m)}}$$

where S and I represent the concentration of substrate and inhibitor, respectively, and α is the factor change to the K_m when the inhibitor is bound to the enzyme-substrate complex. The mode of inhibition which best described the data was determined by comparing R^2 values for the fit of the various models.

Measurements described as “duplicate” or “triplicate” refer to samples analyzed within the same experiment; separate experiments are described as such. All data are expressed as means $\pm$ standard deviation. Two-way analysis of variance (ANOVA) was used to determine differences between the inhibitor-treated and control groups in preincubation studies. Significant interactions between preincubation time and presence of inhibitor were used to demonstrate that the loss in enzyme activity following preincubation was greater than would be expected due to either of these factors alone. To compare K_i values obtained by several different methods, a one-way ANOVA was used. Statistical analyses were performed using SigmaStat for Windows, version 1.0 (Jandel Inc., San Rafael CA, 1994). The level of significance was set, *a priori*, at $p < 0.05$.

Chapter 3

RESULTS

3.1 Assay Validation

HPLC is a commonly used technique for the detection of dextromethorphan and its metabolites. The HPLC assay used in this thesis was adapted from previously published methods. The chromatographic conditions of von Moltke *et al.* (1998) were used, with the organic component of the mobile phase changed from acetonitrile to methanol to achieve better separation of the analytes. The use of fluorescence detection was established by Lam and Rodriguez (1993). Before the assay was used quantitatively, it was validated with respect to its selectivity, linearity of response, limits of quantitation, accuracy, and precision.

The selectivity of an assay defines whether the response from a single analyte can be distinguished from that of other analytes and the biological matrix (Karnes *et al.* 1991). Peaks corresponding to pure standards of dextrophan, 3-hydroxymorphinan, dextromethorphan, and 3-methoxymorphinan were eluted with retention times of 4, 5, 12, and 16 minutes, respectively (figure 4, chromatogram 1). Blank samples containing either microsomal protein (0.5 mg/mL) or recombinant CYP2D6 (2.5 pmol P450/mL) produced no response within the elution windows of any of the four analytes (figure 4, chromatograms 2 and 3). Incubation of either human liver microsomes or recombinant CYP2D6 with dextromethorphan resulted in the generation of peaks corresponding to

dextrorphan and 3-methoxymorphinan, which had similar retention characteristics to the pure metabolite standards (figures 5A and 5B). With recombinant CYP2D6, 3-methoxymorphinan was only detected in incubations with high concentrations of dextromethorphan or, as in figure 5B, enzyme. With the range of substrate concentrations and incubation conditions used experimentally, 3-hydroxymorphinan was not produced.

In order to use an assay quantitatively, a predictable, linear response must be established between the amount of analyte present in the samples and the instrument response (Causon 1997). The calibration curves for each metabolite were constructed by plotting analyte concentration against the integrated peak area (figure 6), and the equation of the line of best fit was determined using unweighted linear regression. The response was found to be linear over the range of 0.1 to 5 μM , with R^2 values greater than 0.99 for all three metabolites.

Figure 4: Chromatographic separation of pure standards of dextromethorphan and its metabolites. Chromatogram 1: resolution of a mixture of the three metabolite standards and dextromethorphan. The peaks correspond to dextromethorphan (DOR), 3-hydroxymorphinan (3-HM), dextromethorphan (DEX) and 3-methoxymorphinan (3-MM). Chromatograms 2 and 3 represent blank microsomal and recombinant CYP2D6 matrix, respectively. Chromatographic conditions were the same as those described in the *Materials and Methods* section 2.3.1.

Figure 5A: Representative chromatogram of dextrorphan and 3-methoxymorphinan produced in a microsomal incubation. Pooled human liver microsomes (0.5 mg/mL) were incubated with 7.5 μ M dextromethorphan and 1 mM NADPH for 20 minutes.

Figure 5B: Representative chromatogram of dextrorphan and 3-methoxymorphinan produced in an incubation with recombinant CYP2D6. Recombinant CYP2D6 (1.5 pmol/mL) was incubated with 10 μ M dextromethorphan and 1 mM NADPH for 15 minutes.

Figure 6: Representative calibration curves for (A) dextrorphan (DOR), (B) 3-hydroxymorphinan (3-HM), and (C) 3-methoxymorphinan (3-MM). Each point is a mean of three measurements. The line of best fit was determined by unweighted linear regression.

The lowest concentration standard included in the calibration curves corresponded to the limit of quantitation (LOQ) of the assay. The LOQ is the lowest concentration of analyte than can be measured with acceptable accuracy and precision. Conventionally, acceptable accuracy is defined as a % bias within 20% of the nominal concentration, while for acceptable precision, the coefficient of variation should not be greater than 20% (Shah *et al.* 1992). For all three metabolites, the lowest concentration which fulfilled these requirements was 0.1 μM .

The accuracy and precision of the assay were determined by measuring the %bias and %CV, respectively, of the QC samples. QC samples corresponding to the high, middle, and low range of the calibration curve were prepared at 4 μM , 2.5 μM , and 0.1 μM , respectively, and included either inactivated microsomal protein or recombinant CYP2D6. The concentrations of the QC samples were determined using calibration curves analyzed at the same time, which also included the appropriate matrix. The variability of the assay is considered acceptable if the % CV is less than 15% and the % bias is within 15% of the nominal concentration; however, because the low QC sample represented the LOQ, a % CV and % bias of less than 20% was considered acceptable (Shah *et al.* 1992). Both the intra- and inter-assay variability were acceptable when either microsomal protein (tables 1 and 2) or recombinant CYP2D6 (tables 3 and 4) was used as the matrix.

Table 1: Intra-assay precision and accuracy of quality control (QC) samples prepared with human liver microsomal matrix. Five QC samples at each of the low (0.1 μ M), mid (2.5 μ M), and high (4 μ M) concentrations were analyzed in a single run. The QC samples were quantitated using calibration curves for the respective metabolites, which were prepared in triplicate and analyzed at the same time. Precision (%CV) and accuracy (%bias) were assessed using the equations described in the *Materials and Methods* section 2.3.3.

Replicate	DOR (μ M)			3-HM (μ M)			3-MM (μ M)		
	QC low	QC mid	QC high	QC low	QC mid	QC high	QC low	QC mid	QC high
1	0.09	2.60	3.80	0.10	2.59	3.81	0.11	2.67	4.19
2	0.09	2.61	4.03	0.10	2.60	4.03	0.11	2.70	4.23
3	0.09	2.61	4.17	0.10	2.60	4.15	0.11	2.70	4.41
4	0.10	2.56	4.16	0.10	2.50	4.14	0.11	2.42	3.85
5	0.10	2.56	4.14	0.10	2.50	4.14	0.11	2.55	3.93
mean	0.09	2.59	4.06	0.10	2.56	4.06	0.11	2.61	4.12
nominal conc'n (μM)	0.10	2.50	4.00	0.10	2.50	4.00	0.10	2.50	4.00
standard deviation	0.00 ^a	0.03	0.16	0.00 ^b	0.05	0.15	0.00 ^c	0.12	0.23
%CV	3.70	1.02	3.83	1.89	2.12	3.60	0.99	4.69	5.60
%bias	-5.53	3.50	1.54	-1.13	2.28	1.38	9.84	4.35	3.07

^a actual value 0.003, rounded to 0.00; ^b actual value 0.002, rounded to 0.00; ^c actual value 0.001, rounded to 0.00.

Table 2: Inter-assay precision and accuracy of quality control (QC) samples prepared with human liver microsomal protein matrix. Five replicates of QC samples at the low (0.1 μ M), mid (2.5 μ M), and high (4 μ M) concentrations were prepared and analyzed along with a calibration curve (in triplicate) each day for five days. Inter-assay precision and accuracy were determined by calculating the %CV and %bias, respectively, of the mean concentration measured on each of the five days.

Day	Average DOR (μ M)			Average 3-HM (μ M)			Average 3-MM (μ M)		
	QC low	QC mid	QC high	QC low	QC mid	QC high	QC low	QC mid	QC high
1	0.10	2.56	4.08	0.10	2.56	4.09	0.10	2.61	4.17
2	0.11	2.59	4.14	0.11	2.60	4.14	0.11	2.68	4.29
3	0.10	2.55	4.59	0.11	2.56	4.48	0.12	2.56	4.59
4	0.12	2.56	4.05	0.11	2.55	4.05	0.12	2.56	4.05
5	0.09	2.60	4.23	0.09	2.59	4.24	0.11	2.61	4.23
mean	0.10	2.57	4.22	0.11	2.57	4.20	0.11	2.60	4.27
nominal conc'n (μ M)	0.10	2.50	4.00	0.10	2.50	4.00	0.10	2.50	4.00
standard deviation	0.01	0.02	0.22	0.01	0.02	0.17	0.01	0.05	0.20
%CV	9.10	0.78	5.26	8.29	0.75	4.08	6.05	1.93	4.71
%bias	4.30	2.84	5.45	5.18	2.82	5.00	11.63	4.11	6.68

Table 3: Intra-assay precision and accuracy of quality control (QC) samples prepared with inactivated recombinant human CYP2D6. Five QC samples at each of the low (0.1 μ M), mid (2.5 μ M), and high (4 μ M) concentrations were analyzed in a single run. The QC samples were quantitated using calibration curves for the respective metabolites, which were prepared in triplicate and analyzed at the same time. Precision (%CV) and accuracy (%bias) were assessed using the equations described in the *Materials and Methods* section 2.3.3.

Replicate	DOR (μ M)		
	QC low	QC mid	QC high
1	0.10	2.63	4.19
2	0.10	2.52	4.03
3	0.10	2.55	4.04
4	0.09	2.54	3.99
5	0.09	2.55	4.01
mean	0.09	2.56	4.05
nominal conc'n (μ M)	0.10	2.50	4.00
standard deviation	0.00 ^a	0.04	0.08
%CV	4.39	1.67	2.00
%bias	-4.13	2.33	1.28

^a actual value 0.004, rounded to 0.00

Table 4: Inter-assay precision and accuracy of quality control (QC) samples prepared with inactivated recombinant human CYP2D6. Five replicates of QC samples at the low (0.1 μM), mid (2.5 μM), and high (4 μM) concentrations were prepared and analyzed along with a calibration curve (in triplicate) each day for five days. Inter-assay precision and accuracy were determined by calculating the %CV and %bias, respectively, of the mean concentration measured on each of the five days.

Day	Average DOR (μM)		
	QC low	QC mid	QC high
1	0.10	2.56	4.05
2	0.12	2.56	4.01
3	0.09	2.56	4.06
4	0.09	2.57	4.09
5	0.10	2.50	3.94
mean	0.10	2.55	4.03
nominal conc'n (μM)	0.10	2.50	4.00
standard deviation	0.01	0.03	0.06
%CV	12.64	1.19	1.42
%bias	-2.26	1.96	0.75

3.2 Optimization of incubation conditions

Incubation conditions for human liver microsomes were optimized with respect to protein concentration, incubation time, and NADPH concentration; while the conditions used for the recombinant enzyme were optimized for P450 concentration, incubation time, and NADPH concentration. Protein or P450 concentrations and incubation times were selected to be within the linear range of product formation and the NADPH concentration was chosen so as not to be a limiting factor in the reactions.

The formation of dextrorphan and 3-methoxymorphinan were linear with microsomal protein concentrations up to 1.0 mg/mL (figure 7). A protein concentration of 0.5 mg/mL was selected, as it was the mid-point of the linear range for dextrorphan formation, which is the metabolite of interest in this thesis. Incubation times of up to 30 minutes produced a linear response for dextrorphan formation, while the formation of 3-methoxymorphinan was linear for only 25 minutes (figure 8). The secondary metabolite, 3-hydroxymorphinan, was not detected with incubation times less than 25 minutes. In order to maximize the formation of dextrorphan, while minimizing the formation of the secondary metabolite, an incubation time of 20 minutes was used. There was no difference in the amount of dextrorphan or 3-methoxymorphinan formed with NADPH concentrations from 0.1 to 2 mM (figure 9). A final NADPH concentration of 1 mM was used to ensure that NADPH was in excess.

With the recombinant enzyme, the formation of dextrorphan was linear with CYP2D6 concentrations of 0.25 to 1 pmol/mL (figure 10) and incubation times up to 20 minutes (figure 11). There was no difference in the amount of dextrorphan formed with NADPH concentrations up to 2 mM (figure 12). A final P450 concentration of 0.5 pmol/mL, an incubation time of 15 minutes, and an NADPH concentration of 1 mM were used in all subsequent incubations. Under these conditions, the formation of 3-hydroxymorphinan was not detectable.

Figure 7: Optimization of microsomal protein concentration. Pooled human liver microsomes (0.15 to 1 mg protein/mL) were incubated with 10 μ M dextromethorphan and 1 mM NADPH for 20 minutes. The amount of dextrophan (DOR) formed is shown in the top panel, while 3-methoxymorphinan (3-MM) is shown in the bottom panel. Each point represents the mean of two measurements.

Figure 8: Optimization of incubation time with pooled human liver microsomes. Microsomal protein (0.5 mg/mL) was incubated with dextromethorphan (10 μ M) and NADPH (1 mM) for 2.5 to 120 minutes. The top panel shows the formation of dextrorphan (DOR) with time, while the bottom shows the formation of 3-methoxymorphinan (3-MM). Each point represents the mean of duplicate measurements.

Figure 9: Optimization of NADPH concentration with human liver microsomes. Pooled human liver microsomes (0.5 mg protein/mL) were incubated with 10 μ M dextromethorphan and various concentrations of NADPH (0.1 to 5 mM) for 20 minutes. The amount of dextrorphan (DOR, top panel) and 3-methoxymorphinan (3-MM, bottom panel) formed was measured in duplicate at each point.

Figure 10: Optimization of recombinant CYP2D6 concentration. Recombinant CYP2D6 (0.25 to 5 pmol/mL) was incubated with 5 μ M dextromethorphan and 1 mM NADPH for 15 minutes and the amount of dextrophan (DOR) formed was measured. Each point represents the mean of two measurements.

Figure 11: Optimization of incubation time with recombinant CYP2D6. Recombinant CYP2D6 (0.5 pmol/mL) was incubated with 5 μ M dextromethorphan and 1 mM NADPH for 5 to 120 minutes and the amount of dextrophan (DOR) formed was measured. Each point represents the mean of two measurements

Figure 12: Optimization of NADPH concentration with recombinant CYP2D6. Recombinant CYP2D6 (0.5 pmol/mL) was incubated with 5 μ M dextromethorphan and NADPH concentrations ranging from 0.1 to 2 mM for 15 minutes and the amount of dextrorphan (DOR) formed was measured. Each point is the mean of two measurements.

3.3 Studies with human liver microsomes

The effect of cimetidine on CYP2D6 activity was first determined using human liver microsomes. This *in vitro* model is more representative of the metabolic conditions seen in the liver *in vivo*, as the cytochrome P450 enzymes are present in their normal lipid environment and the accessory proteins, such as NADPH-P450 reductase, are present in normal proportions. Also, a full complement of hepatic P450 enzymes is present. While this leads to a more complex system, where several enzymes may be contributing to the probe activity, use of appropriate substrate concentrations can increase the specificity of the reaction of interest. When investigating the potential formation of a catalysis-dependent inhibitory species, the presence of all P450 enzymes is beneficial because the inhibitory species could possibly be formed by an enzyme other than the one which is targeted. Therefore, by using a model in which all enzymes are present, the likelihood of developing the conditions under which a catalysis-dependent increase in inhibitory potency can be observed is improved.

3.3.1 Kinetic constants of dextromethorphan O-demethylation

The formation of dextrorphan, the O-demethylated metabolite of dextromethorphan, was used as a marker of CYP2D6 activity. The apparent K_m and V_{max} of dextrorphan formation were estimated with pooled human liver microsomes by fitting data to the Michaelis-Menten equation (Figure 13). The apparent K_m was determined to be $4.1 \pm 0.1 \mu M$, while the V_{max} was $0.82 \pm 0.06 \text{ nmol/min/nmol P450}$ in three trials (Table 5). The K_m value determined from this experiment was used to select appropriate substrate concentrations in subsequent inhibition studies. The Eadie-Hofstee plot was linear over the range of substrate concentrations used to determine the kinetic constants (figure 13B), suggesting that the observed activity is predominantly representative of a single enzyme.

Figure 13: Rate of formation of dextromethorphan by human liver microsomes. Pooled human liver microsomes (0.5 mg protein/mL) were incubated with dextromethorphan (0.5 to 60 μM) and NADPH (1 mM) for 20 minutes. Panel A: data were fit to the Michaelis-Menten equation using non-linear regression. Panel B: Eadie-Hofstee transformation of the data presented in panel A. Each point is the mean of duplicate measurements.

Table 5: Summary of the kinetic constants for dextromethorphan *O*-demethylation by pooled human liver microsomes determined in three separate experiments.

Trial	$V_{\max}$ (nmol/min/nmol P450)	K_m (μM)	$V_{\max}/K_m$
1	0.86	4.0	0.22
2	0.84	4.2	0.20
3	0.75	4.2	0.18
mean	0.82	4.1	0.20
standard deviation	0.06	0.1	0.02

3.3.2 Effect of quinidine and cimetidine on dextromethorphan O-demethylase activity

Quinidine is a known competitive inhibitor of CYP2D6 (Kobayashi *et al.* 1989). Pooled human liver microsomes were incubated with various concentrations of inhibitor, at three fixed concentrations of dextromethorphan (approximately 0.5-, 1-, and 2-times the K_m), and the K_i value was estimated to be 52.6 ± 28.2 nM (figure 14, panel A). A competitive inhibition model provided the best fit for the data. A representative Lineweaver-Burk plot is shown in figure 14, panel B.

Two forms of cimetidine, the hydrochloride salt and the free base, were available for use in the inhibition studies. Cimetidine hydrochloride was obtained from the manufacturer, SmithKline & French Canada (Mississauga ON), in 1988 and was used in previous studies in this laboratory which demonstrated that cimetidine acts as an *in vitro* mechanism-based inactivator of rat CYP2C11 (Chang *et al.* 1992). However, because of the age of the product, there was a possibility that it may have undergone significant chemical degradation. Cimetidine has been shown to be stable when stored at room temperature in a dry environment for up to five years (Bavin *et al.* 1984), but there is no data to support its stability beyond this period of time. The free base, on the other hand, was readily available from a commercial source. Dose response curves for the two forms of cimetidine are shown in figure 15. The curve for the hydrochloride salt is shifted to the right compared to the curve for the free base, but the IC_{50} values are similar (approximately 100 μ M for the free base, 150 μ M for cimetidine hydrochloride, as determined by visual inspection of the curves). Because there was little difference between the two forms, the free base, which was more easily obtained, was used in all subsequent experiments.

The K_i for inhibition of dextromethorphan O-demethylase activity by cimetidine was determined using a similar method to that used with quinidine, and was found to be 38.0 ± 5.3 μ M. As with quinidine, a competitive inhibition model provided the best fit for the data (figure 16). The individual K_i values for quinidine and cimetidine, determined in three separate trials, are summarized in table 6.

Figure 14: Inhibition of dextromethorphan *O*-demethylase activity in human liver microsomes by quinidine. **A:** Dixon plot. Pooled human liver microsomes (0.5 mg protein/mL) were incubated with dextromethorphan (dex, 2.5, 4.5, or 7.5 μM), quinidine (0.025 to 0.2 μM), and NADPH (1 mM) for 20 minutes. Data were fit to a competitive inhibition model using the SigmaPlot enzyme kinetics module. Each point represents the mean of duplicate measurements. **B:** Lineweaver-Burk transformation of the data in panel A.

Figure 15: Effect of cimetidine free base and hydrochloride salt on dextromethorphan *O*-demethylase activity in human liver microsomes. Pooled human liver microsomes (0.5 mg protein/mL) were incubated with dextromethorphan (10 μM), cimetidine free base or hydrochloride salt (10 to 1000 μM), and NADPH (1 mM) for 20 minutes. Percent control activity was determined by comparing the activity in the samples treated with inhibitor to that of a group incubated with dextromethorphan (10 μM) and NADPH (1 mM) alone. The activity of the control groups were 0.458 and 0.493 nmol/min/nmol P450, for cimetidine free base and hydrochloride salt, respectively. Each point represents the mean of two experiments in which duplicate measurements were made.

Figure 16: Inhibition of dextromethorphan *O*-demethylase activity in human liver microsomes by cimetidine. A: Dixon plot. Pooled human liver microsomes (0.5 mg protein/mL) were incubated with dextromethorphan (dex, 2.5, 4.5, or 7.5 μM), cimetidine (25 to 250 μM), and NADPH (1 mM) for 20 minutes. Data were fit to a competitive inhibition model using the SigmaPlot enzyme kinetics module. Each point represents the mean of duplicate measurements. B: Lineweaver-Burk transformation of the data presented in panel A.

Table 6: Summary of estimated K_i values for inhibition of dextromethorphan O-demethylation by quinidine and cimetidine with pooled human liver microsomes, determined in three separate experiments. The K_i values for quinidine and cimetidine were estimated using a competitive inhibition model.

Trial	apparent K_i	
	quinidine (nM)	cimetidine (μM)
1	77.6	32.2
2	22.0	42.6
3	58.2	39.2
mean	52.6	38.0
standard deviation	28.2	5.3

3.3.3 Preincubation studies with cimetidine

In order to determine whether cimetidine was capable of acting as a mechanism-based inactivator of CYP2D6, human liver microsomes were preincubated with cimetidine and NADPH for various periods of time before measuring the dextromethorphan *O*-demethylase activity. The method used for the preincubation studies involved two steps: a preincubation reaction with the inhibitor and incubation reaction with the substrate. The microsomal protein concentration in the preincubation reaction was 2 mg/mL, four times higher than the optimized concentration for incubations with dextromethorphan. At appropriate time points, an aliquot of the preincubation mixture was added to a reaction tube containing substrate and NADPH with a four-fold dilution, resulting in a microsomal protein concentration of 0.5 mg/mL in the final incubation. This dilution step also had the effect of reducing the concentration of cimetidine in the incubation to one-fourth of what was used in the preincubation reaction. Because cimetidine was demonstrated to act as a competitive inhibitor (see section 3.3.2), any cimetidine carried over from the preincubation reaction to the incubation reaction could potentially cause reversible inhibition of CYP2D6 activity. However, the observation of mechanism-based inactivation, not competitive inhibition, was the goal of the following experiments. Using this method for the preincubation experiments minimized the competitive inhibition of dextromethorphan *O*-demethylase activity by cimetidine during the incubation reaction.

Initially, pooled human liver microsomes were preincubated with 25 μ M cimetidine for up to 30 minutes. Without preincubation, there was a 25% inhibition of activity, which was likely due to competitive inhibition by the residual cimetidine in the incubation reaction. With 5 to 30 minutes of preincubation, there was no trend towards a further decrease in dextromethorphan *O*-demethylase activity (table 7)

Table 7: Preincubation of human liver microsomes with cimetidine (25 μ M) for up to 30 minutes. Pooled human liver microsomes (2 mg protein/mL) were preincubated with cimetidine (25 μ M) and NADPH (1 mM), or with NADPH alone (control). At 10, 20, and 30 minutes after initiation of the preincubation reaction, a 50 μ L aliquot was removed from the preincubation mixture and added to a new reaction tube containing dextromethorphan and NADPH. Samples were incubated for a further 20 minutes. In the final incubation step, the concentration of dextromethorphan was 5 μ M and the concentration of microsomal protein was 0.5 mg/mL. Data shown are the mean of duplicate measurements. Activity in the cimetidine-treated groups was normalized to the activity of the control group preincubated for the same period of time to give the % control activity.

preincubation time (minutes)	dextromethorphan O-demethylation (nmol/min/nmol P450)		% control activity
	control	cimetidine	
0	0.42	0.32	75%
10	0.36	0.30	83%
20	0.35	0.30	86%
30	0.37	0.30	81%

To investigate whether a trend toward increasing inhibition could be observed with longer preincubation times, the preincubation period was increased to 120 minutes. Over this longer period of time, the preincubation mixture was supplemented with NADPH every 30 minutes to ensure that the cofactor did not become a limiting factor in the preincubation reaction. Under these conditions, however, activity of the groups preincubated with cimetidine and NADPH, or NADPH alone, decreased by 72.6% and 84.7%, respectively, after a period of 120 minutes (figure 17). In contrast, activity in the group to which neither NADPH nor cimetidine were added dropped by only 17.7% over the same period of time.

Because the drop in activity appeared to be related to the addition of excess NADPH, the conditions under which sufficient NADPH was added so as not to become the limiting reagent, but also to not cause excessive loss of microsomal activity, were investigated (table 8). Microsomes were incubated with either 25 μ M or 100 μ M cimetidine for 120 minutes. NADPH was added to initiate the preincubation reaction, and was then added again after 60 minutes (+NADPH), or was only added at the beginning of the preincubation period (-NADPH). In both cases, the loss of control activity was approximately 30% after 120 minutes of preincubation. The absolute activity was slightly, but significantly, higher in the groups to which NADPH was added only once; however, the % control activities of the cimetidine-treated groups were similar after 120 minutes of preincubation whether extra NADPH was added at 60 minutes or not. These results suggest that adding NADPH to the preincubation mixture only once does not limit the reaction of cimetidine with CYP2D6 and maintains a high level of enzyme activity.

Figure 17: Effect of addition of NADPH to the preincubation mixture every 30 minutes. Pooled human liver microsomes (2 mg protein/mL) were preincubated with NADPH (1 mM), and 25 μ M cimetidine (+cimetidine+NADPH) or an equal volume of buffer (+NADPH). Every 30 minutes, a 50 μ L aliquot was removed from the preincubation mixture and added to a reaction tube containing dextromethorphan and NADPH in a volume of 150 μ L. In the final incubation, the concentration of microsomal protein was 0.5 mg/mL and dextromethorphan was 5 μ M. Every 30 minutes, immediately following the removal of the 50 μ L aliquots, an amount of NADPH equivalent to a final concentration of 0.5 mM was added to the preincubation mixture. A control preincubation mixture, which consisted of pooled human liver microsomes (2 mg/mL) and incubation buffer alone, and to which NADPH was added only at the beginning of the preincubation period, was run in parallel. Data shown are the mean of four measurements $\pm$ standard deviation. # activity is significantly ($p < 0.05$) lower than that of control group preincubated for the same period of time and groups preincubated with NADPH alone for 0, 30, 60, and 90 minutes. * activity is significantly ($p < 0.05$) lower than that of group preincubated with NADPH alone for the same period of time and groups preincubated with cimetidine and NADPH for 0, 30, 60, and 90 minutes.

Table 8: Effect of adding NADPH to the preincubation mixture every 60 minutes versus only once at the beginning of the preincubation reaction. Pooled human liver microsomes (2 mg/mL) were preincubated with cimetidine (25 or 100 μ M), or an equal volume of buffer (control). NADPH (1 mM) was added at the beginning of the preincubation period and again after 60 minutes (+NADPH), or just at the beginning of the preincubation period (-NADPH). The concentration of dextromethorphan in the incubation was 5 μ M. Data shown are the mean of 3 measurements $\pm$ standard deviation (% control activity). % control activity was determined by normalizing the activity of the cimetidine-treated groups to the activity of the control group preincubated for the same duration of time. *significantly different ($p < 0.05$) from corresponding +NADPH groups.

preincubation time (minutes)	+NADPH			-NADPH		
	control	25 μ M cimetidine	100 μ M cimetidine	control	25 μ M cimetidine	100 μ M cimetidine
0	0.325$\pm$0.006	0.292 $\pm$ 0.003 (89.8%)	0.285 $\pm$ 0.004 (87.7%)	0.328$\pm$0.006	0.322 $\pm$ 0.040 (98.2%)	0.288 $\pm$ 0.006 (87.8%)
120	0.217$\pm$0.007	0.237 $\pm$ 0.003 (109.2%)	0.215 $\pm$ 0.001 (99.1%)	0.230$\pm$0.002*	0.245 $\pm$ 0.001* (106.5%)	0.241 $\pm$ 0.004* (104.8%)

After optimization of the conditions for the longer preincubation period, the experiment was repeated using pooled human liver microsomes. Microsomes were also obtained from two individuals who had relatively high CYP2D6 activity as assessed by bufuralol 1-hydroxylase activity (activity in the pooled microsomes was 120 pmol product/minute/mg protein, while for individual HG 95 and HG 64, activity was 160 and 220 pmol product/minute/mg protein, respectively), as measured by the supplier. Two concentrations of cimetidine, 25 μ M and 100 μ M, were used in each experiment. The 25 μ M concentration represents an amount of inhibitor that should produce little or no competitive inhibition in the incubation step. After the dilution step, the concentration of cimetidine in the final incubation was 6.25 μ M, much lower than the K_i for competitive inhibition (which was found to be 38.0 μ M, as discussed in section 3.3.2). Any inactivation of CYP2D6 should be more apparent under these conditions because the loss in activity due to competitive inhibition alone would potentially be small compared to the effect of enzyme inactivation. The second concentration (100 μ M) was selected in order to increase the effect of the inactivation reaction, if such an effect were occurring. Because the concentration of cimetidine in the incubation step approached the K_i in this case, carrying out the preincubation reaction with more than 100 μ M cimetidine was impractical.

The results from the pooled human liver microsomes are shown in figure 18. Preincubation for 120 minutes with NADPH alone resulted in a decrease in activity of approximately 30%. With 25 μ M cimetidine, a 10% decrease in activity was seen without any preincubation, and with preincubation times of 30 to 120 minutes no difference in activity was seen in the cimetidine-treated groups compared to the appropriate control. With 100 μ M cimetidine, an inhibition of 26.4% was seen without preincubation, which represents the competitive inhibition caused by the presence of 25 μ M cimetidine in the final incubation step. When preincubated from 30 to 120 minutes, the activity in the 100 μ M cimetidine-treated group remained lower than that of the control group at each time point, but there was no trend towards increasing loss of activity with time, as shown in the lack of change in % control activity.

Figure 18: Preincubation of pooled human liver microsomes with cimetidine for up to 120 minutes. Pooled human liver microsomes (2 mg protein/mL) were preincubated with cimetidine (25 μ M or 100 μ M) or incubation buffer (control) and NADPH (1 mM). At 30 minute intervals, 50 μ L of the preincubation mixture was removed and added to a reaction tube containing dextromethorphan and NADPH, in a volume of 150 μ L, and incubated for a further 20 minutes. In the final incubation, the concentration of dextromethorphan was 5 μ M and the concentration of microsomal protein was 0.5 mg/mL. Each point represents the mean of triplicate measurements $\pm$ standard deviation. * significantly lower ($p < 0.05$) than control group preincubated for the same period of time.

With the microsomes from the individuals HG 95 (figure 19) and HG 64 (figure 20), the amount of inhibition seen without preincubation was similar to that seen with the pooled human liver microsomes with both 25 and 100 μM cimetidine. Again, there was no trend toward increasing inhibition with longer preincubation times at either concentration of cimetidine. In addition, the activity of the control groups decreased more rapidly than that of the cimetidine-treated groups, thus masking any inhibitory effect cimetidine might have been having.

Because preincubation with cimetidine did not result in an increase in inhibition under the conditions used in the experiments described above, the effect of using a saturating concentration of substrate in the incubation step was investigated. Microsomes from individual HG 64 were preincubated with 100 μM cimetidine for 90 minutes and then incubated with 50 μM dextromethorphan, a concentration roughly 10-times the K_m . Under conditions where a saturating substrate concentration was used, the competitive inhibition by the cimetidine present in the incubation reaction should be minimized, therefore a decrease in activity seen following preincubation should be exclusively due to mechanism-based inactivation. Using a higher substrate concentration also provided information regarding the *N*-demethylation pathway of dextromethorphan metabolism, which has a lower affinity compared to the *O*-demethylation pathway. A second substrate concentration (300 μM), which approximated the K_m of the *N*-demethylation pathway (von Moltke *et al.* 1998) was also included. Microsomes from individual HG 64 were used because this sample demonstrated the highest dextromethorphan *O*-demethylase activity. Additionally, in the experiment described in figure 20, there appeared to be a slight trend towards increasing inhibition of 3-methoxymorphinan formation by cimetidine with preincubation, which was most apparent after 90 minutes of preincubation; however, because of the low substrate concentration used in that experiment, the formation of 3-methoxymorphinan was too low to be accurately quantitated.

As expected, without preincubation the activity in the cimetidine-treated group was the same as that of the control group (figure 21), indicating that at higher substrate concentrations the competitive

inhibition of cimetidine was overcome. There was no increase in cimetidine's inhibitory effect on dextrorphan formation with preincubation. For the *N*-demethylase pathway, there was an 11.5% drop in activity in the cimetidine-treated group preincubated for 90 minutes compared to the control group preincubated for the same period of time, when the incubation reaction contained 50 μ M dextromethorphan (figure 22A). There was no observable inhibition without preincubation. At the higher substrate concentration (figure 22B), there was also no observable inhibition, either with or without preincubation. Because the inhibitory effect seen at the lower substrate concentration was small and the *N*-demethylation pathway involves several CYP enzymes, thus making it difficult to determine which enzyme is involved in the inhibition, it was not investigated further.

Figure 19: Preincubation of human liver microsomes from individual HG 95 with cimetidine for up to 120 minutes. Microsomal protein (2 mg/mL) was preincubated with cimetidine (25 μ M or 100 μ M), or incubation buffer (control), and NADPH (1 mM). At 30 minute intervals, 50 μ L of the preincubation mixture was removed and added to a reaction tube containing dextromethorphan and NADPH, in a volume of 150 μ L, and incubated for a further 20 minutes. In the final incubation, the concentration of dextromethorphan was 5 μ M, and the concentration of microsomal protein was 0.5 mg/mL. Each point represents the mean of triplicate measurements $\pm$ standard deviation. * significantly lower ($p < 0.05$) than control group preincubated for the same period of time. # significantly lower than the control group preincubated for the same period of time and the group treated with 100 μ M cimetidine that was not preincubated.

Figure 20: Preincubation of human liver microsomes from individual HG 64 with cimetidine for up to 120 minutes. Microsomal protein (2 mg/mL) was preincubated with cimetidine (25 μM or 100 μM) or incubation buffer (control) and NADPH (1 mM). At 30 minute intervals, 50 μL of the preincubation mixture was removed and added to a reaction tube containing dextromethorphan and NADPH, in a volume of 150 μL, and incubated for a further 20 minutes. In the final incubation, the concentration of dextromethorphan was 5 μM, and the concentration of microsomal protein was 0.5 mg/mL. Each point represents the mean of triplicate measurements $\pm$ standard deviation. * activity is significantly lower ($p < 0.05$) than control group preincubated for the same period of time.

Figure 21: Effect of preincubation of human liver microsomes with cimetidine for 90 minutes, followed by incubation with 50 μ M dextromethorphan, on dextromethorphan *O*-demethylase activity in human liver microsomes. Microsomal protein (2 mg/mL) from donor HG64 was preincubated with cimetidine (100 μ M) and NADPH (1 mM). Before initiation of the preincubation reaction, and 90 minutes later, a 50 μ L aliquot was removed from the preincubation mixture and added to a reaction tube containing dextromethorphan and NADPH, in a volume of 150 μ L. Samples were then incubated for a further 20 minutes. The final concentration of dextromethorphan in the incubation reaction was 50 μ M. Each bar represents the mean of triplicate measurements + standard deviation. No statistically significant inhibition was seen in either of the cimetidine-treated groups.

Figure 22: Effect of preincubation of human liver microsomes with cimetidine for 90 minutes, followed by incubation with 50 μ M or 300 μ M dextromethorphan, on dextromethorphan *N*-demethylase activity of human liver microsomes. Microsomal protein (2 mg/mL) from donor HG64 was preincubated with cimetidine (100 μ M) and NADPH (1 mM). Before initiation of the preincubation reaction, and 90 minutes later, a 50 μ L aliquot was removed from the preincubation mixture and added to a reaction tube containing dextromethorphan and NADPH, in a volume of 150 μ L. Samples were then incubated for a further 20 minutes. The final concentration of dextromethorphan in the incubation reaction was 50 μ M or 300 μ M. Each bar represents the mean of 3 measurements + standard deviation. * significantly different ($p < 0.05$) from control group preincubated for 90 minutes and from cimetidine-treated group which was not preincubated.

3.3.4 Mechanism-based inactivation with triacetylleandomycin and 1-aminobenzotriazole

Two compounds known to act as mechanism-based inactivators, triacetylleandomycin and 1-aminobenzotriazole, were used as positive controls to demonstrate that this type of inhibition could be observed under the same conditions used for the cimetidine preincubation studies.

Triacetylleandomycin has been shown to be a specific inactivator of CYP3A (Chang *et al.* 1994). Without preincubation, triacetylleandomycin inhibited the *N*-demethylation of dextromethorphan, a pathway partially mediated by CYP3A, by 27.4%. Following preincubation with NADPH for 30 minutes, a 45.7% decrease in activity was observed (figure 23).

In contrast to triacetylleandomycin, 1-aminobenzotriazole is a more general P450 inactivator and has been shown to inactivate a number of enzymes, including CYP2D6 (Balani *et al.* 2002). A more specific CYP2D6 inactivator could not be found; however, using incubation conditions under which the formation of dextromethorphan is primarily mediated by CYP2D6, mechanism-based inactivation was demonstrated. Following 30 minutes of preincubation with NADPH, 1-aminobenzotriazole decreased the formation of dextromethorphan by 34%, compared to no observable loss in activity without preincubation (figure 24).

3.3.5 Summary of studies with human liver microsomes

Both cimetidine and quinidine were shown to be competitive inhibitors of dextromethorphan *O*-demethylation. Preincubation with NADPH and cimetidine for up to 120 minutes did not result in a further loss of activity compared to the appropriate control. The lack of observable mechanism-based inactivation by cimetidine under these conditions, however, is not due to any intrinsic properties of the experimental system as positive results were observed with both triacetylleandomycin and 1-aminobenzotriazole.

Figure 23: Inactivation of dextromethorphan *N*-demethylase activity in human liver microsomes by triacetyloleandomycin (TAO). Pooled human liver microsomes (2 mg/mL) were preincubated with 5 μ M TAO, or an equal volume of buffer (control), and NADPH (1 mM). At 0 and 30 minutes after initiation of the preincubation reaction, a 50 μ L aliquot was removed and added to a reaction tube containing dextromethorphan (300 μ M) and NADPH (1 mM), and incubated for a further 15 minutes. In the final incubation step, the concentration of microsomal protein was 0.5 mg/mL and the concentration of TAO was 1.25 μ M. Each bar represents the mean of four measurements + standard deviation. * significantly different ($p < 0.05$) from control group preincubated for the same period of time. There was a significant interaction ($p < 0.05$) between preincubation time and TAO treatment.

Figure 24: Inactivation of dextromethorphan *O*-demethylase activity in human liver microsomes by 1-aminobenzotriazole (ABT). Pooled human liver microsomes (2 mg/mL) were preincubated with 20 μ M ABT, or an equal volume of buffer (control), and NADPH (1 mM). At 0 and 30 minutes after initiation of the preincubation reaction, a 50 μ L aliquot was removed and added to a reaction tube containing dextromethorphan (5 μ M) and NADPH (1 mM), and incubated for a further 15 minutes. In the final incubation step, the concentration of microsomal protein was 0.5 mg/mL and the concentration of ABT was 5 μ M. Each bar represents the mean of four measurements + standard deviation. * significantly different ($p < 0.05$) from the control group preincubated for the same period of time. There was a significant interaction ($p < 0.05$) between preincubation time and ABT treatment.

3.4 Studies with recombinant human CYP2D6

3.4.1 Kinetic constants of dextromethorphan O-demethylation

The $V_{\max}$ and K_m values for dextromethorphan O-demethylation were determined from Michaelis-Menten plots and were estimated to be 15.9 ± 0.8 nmol/min/nmol P450 and 1.4 ± 0.6 μ M, respectively (figure 25). The average ratio of $V_{\max}/K_m$ with the recombinant enzyme was 13.1 (table 9). This same ratio with the human liver microsomes was 0.2 (table 5), indicating that the catalytic efficiency of the recombinant system for this pathway is considerably higher than that of the microsomal system. As with the results from the microsomal experiment, the K_m value determined in this experiment was used to select appropriate concentrations of substrate for subsequent experiments.

3.4.2 Effect of quinidine and cimetidine on dextromethorphan O-demethylase activity

The K_i values for inhibition by quinidine and cimetidine were determined by incubating recombinant CYP2D6 with various concentrations of inhibitor at three fixed concentrations of substrate (approximately 0.5-, 1-, and 2-times the K_m). From Dixon plots, (figure 26A) the K_i value for quinidine was estimated to be 1.3 ± 0.4 nM ($n=3$). The competitive inhibition model best fit the data, as was seen with the human liver microsomes, and a representative Lineweaver-Burk plot is shown in figure 26B. For cimetidine, the model that provided the best fit for the data was that of mixed inhibition, and the estimated K_i value was 102.5 ± 16.8 μ M ($n=3$). Representative Dixon and Lineweaver-Burk plots for cimetidine are shown in figure 27.

The individual K_i values from three independent trials with both quinidine and cimetidine are summarized in table 10.

Figure 25: Rate of formation of dextrorphan by recombinant CYP2D6. Recombinant CYP2D6 (2.5 pmol/mL) was incubated with dextromethorphan (0.25 to 80 μ M) and NADPH (1mM) for 15 minutes. Data were fit to the Michaelis-Menten equation using non-linear regression. Each point represents the mean of duplicate measurements.

Table 9: Summary of the kinetic constants for dextromethorphan *O*-demethylation by recombinant CYP2D6 determined in three separate experiments.

Trial	$V_{\max}$ (nmol/min/nmol P450)	K_m (μM)	$V_{\max}/K_m$
1	15.2	1.0	15.2
2	16.8	1.0	16.8
3	15.6	2.1	7.4
mean	15.9	1.4	13.1
standard deviation	0.8	0.6	5.1

Figure 26: Inhibition of dextromethorphan *O*-demethylase activity of recombinant CYP2D6 by quinidine. Recombinant CYP2D6 (2.5 pmol/mL) was incubated with dextromethorphan (dex, 0.5 to 2 μ M), quinidine (0.5 to 7.5 nM), and NADPH (1 mM) for 15 minutes. Data were fit to a competitive inhibition model using the SigmaPlot enzyme kinetics module. Each point represents the mean of duplicate measurements. A: Dixon plot. B: Lineweaver-Burk transformation of the data presented in panel A.

Figure 27: Inhibition of dextromethorphan *O*-demethylase activity of recombinant CYP2D6 by cimetidine. Recombinant CYP2D6 (2.5 pmol/mL) was incubated with dextromethorphan (dex, 0.5 to 2 μM), cimetidine (25 to 150 μM), and NADPH (1 mM) for 15 minutes. Data were fit to a mixed inhibition model using the SigmaPlot enzyme kinetics module. Each point represents the mean of duplicate measurements. A: Dixon plot. B: Lineweaver-Burk transformation of data in panel A.

Table 10: Summary of estimated K_i values for inhibition of dextromethorphan *O*-demethylation by quinidine and cimetidine with recombinant CYP2D6, determined in three separate experiments. The K_i values for quinidine were determined using a competitive inhibition model, while a mixed inhibition model was used for cimetidine.

Trial	apparent K_i	
	quinidine (nM)	cimetidine (μM)
1	1.4	117.6
2	1.6	105.5
3	0.9	84.3
mean	1.3	102.5
standard deviation	0.4	16.8

3.4.3 Preincubation studies with cimetidine

To determine whether cimetidine could act as a mechanism-based inactivator in the recombinant system, CYP2D6 was preincubated with this inhibitor for up to 20 minutes (figure 28). Following 20 minutes of preincubation, the activity of the control group, which included CYP2D6 and NADPH in the absence of cimetidine, had decreased by 54.3%. Due to the instability of the enzyme activity, the preincubation period was not extended beyond this time. A cimetidine concentration of 50 μM was used in the preincubation reaction, resulting in an inhibitor concentration of 12.5 μM in the final incubation step. This concentration was selected to minimize the amount of competitive inhibition seen, based on the K_i value of 102.5 μM .

In the cimetidine-treated group, there was a trend toward increasing loss of activity with time from 7.2% without preincubation to 34.7% after 20 minutes of preincubation, compared to the control group preincubated with NADPH alone. In addition to being dependent on preincubation time, the loss of activity observed in the cimetidine-treated group was also dependent on the presence of NADPH in the preincubation mixture. In groups that did not include NADPH in the preincubation mixture, an initial drop in activity of 6.4% was seen in the cimetidine treated group compared to the control without preincubation, however, this drop in activity did not increase with longer preincubation times.

To determine the consistency of the effect of preincubation on inhibition with cimetidine, three independent experiments were performed in which CYP2D6 was preincubated with 100 μM cimetidine for 20 minutes. The average activity of the cimetidine-treated group was 35.6% (95% confidence interval, 33.7-37.5) lower than that of the control group after preincubation (figure 29).

Figure 25: Preincubation of recombinant CYP2D6 with cimetidine for 5, 10, and 20 minutes, followed by incubation with 1 μ M dextromethorphan. Recombinant CYP2D6 (10 pmol/mL) was preincubated with cimetidine (50 μ M) and NADPH (1 mM), or NADPH (1 mM) alone. Aliquots (50 μ L) were removed at 5, 10, and 20 minutes following initiation of the preincubation reaction and were added to reaction tubes containing dextromethorphan (1 μ M) and NADPH (1 mM) and reactions were incubated for a further 15 minutes. In the final incubation step, the concentration of recombinant CYP2D6 was 2.5 pmol/mL and the concentration of cimetidine was 12.5 μ M in a volume of 200 μ L. A second set of samples were preincubated with or without cimetidine in the absence of NADPH. Each point represents the mean of three measurements $\pm$ standard deviation. * significantly different from groups preincubated with NADPH alone for the same period of time. # significantly different from groups preincubated with NADPH and cimetidine for 0, 5, and 10 minutes.

Figure 29: Preincubation of CYP2D6 with cimetidine (50 μ M) for 20 minutes, followed by incubation with 1 μ M dextromethorphan. Recombinant CYP2D6 (10 pmol/mL) was preincubated with cimetidine (50 μ M) and NADPH (1 mM), or NADPH alone (control), for 20 minutes. A 50 μ L aliquot was then removed and added to a reaction tube containing dextromethorphan (1 μ M) and NADPH (1 mM), in a volume of 150 μ L. Samples were then incubated for a further 15 minutes. In the final incubation step, the concentration of recombinant CYP2D6 was 2.5 pmol/mL and the concentration of cimetidine was 12.5 μ M. Data shown are the mean of four separate experiments, in which activity was measured in triplicate in each group, + standard deviation. * significantly different from preincubated control and cimetidine-treated group without preincubation ($p < 0.05$).

Despite the presence of a relatively low concentration of cimetidine (12.5 μ M) in the incubation step of these experiments, a small degree of competitive inhibition was still observed, as can be seen by the 7% inhibition in activity without preincubation in figure 28. To eliminate the contribution of competitive inhibition, and to ensure that enzyme inactivation due to preincubation with cimetidine is primarily responsible for the observed decrease in activity, a saturating concentration of dextromethorphan was used in the incubation reaction.

Following 20 minutes of preincubation with 50 μ M cimetidine, dextromethorphan O-demethylase activity was decreased by 10.6% (95% confidence interval, 5.0-16.2; figure 30) when a saturating substrate concentration (equivalent to approximately 20 times the K_m) was used in the incubation reaction. Increasing the cimetidine concentration to 100 μ M resulted in a 17.8% (95% confidence interval, 13.4-22.2) decrease in activity with preincubation (figure 30). In both cases, without preincubation there was no difference in the activity of the cimetidine treated groups compared to that of the control. In addition to being dependent on inhibitor concentration, the decrease in activity was also dependent on preincubation time and the presence of NADPH (figure 31).

Figure 30: Preincubation of CYP2D6 with cimetidine for 20 minutes, followed by incubation with a saturating concentration of dextromethorphan. Recombinant CYP2D6 (10 pmol/mL) was preincubated with 50 μ M (n=3) or 100 μ M (n=4) cimetidine and NADPH (1 mM), or NADPH alone (control), for 20 minutes. A 50 μ L aliquot was then removed and added to a reaction tube containing dextromethorphan (20 μ M) and NADPH (1 mM). Samples were then incubated for a further 15 minutes. In the final incubation step, the concentration of recombinant CYP2D6 was 2.5 pmol/mL and the concentration of cimetidine was 12.5 or 25 μ M, for samples preincubated with 50 or 100 μ M, respectively. Data shown are the mean of three or four separate experiments, in which activity was measured in triplicate in each group, + standard deviation. * significantly different from preincubated control ($p < 0.05$). There was a significant interaction ($p < 0.05$) between preincubation time and treatment with cimetidine.

Figure 31: Preincubation of recombinant CYP2D6 with cimetidine for 5, 10, and 20 minutes, followed by incubation with a saturating concentration of dextromethorphan. Recombinant CYP2D6 (10 pmol/mL) was preincubated with cimetidine (100 μ M) and NADPH (1 mM), or NADPH (1 mM) alone. Aliquots (50 μ L) were removed at 5, 10, and 20 minutes following initiation of the preincubation reaction and were added to reaction tubes containing dextromethorphan (20 μ M) and NADPH (1 mM). Reactions were incubated for a further 15 minutes. In the final incubation step, the concentration of recombinant CYP2D6 was 2.5 pmol/mL and the concentration of cimetidine was 25 μ M in a volume of 200 μ L. A second set of samples were preincubated with or without cimetidine in the absence of NADPH. Each point represents the mean of three measurements $\pm$ standard deviation. * significantly different from groups preincubated with NADPH alone for the same period of time. # significantly different from groups preincubated with NADPH and cimetidine for 0, 5, and 10 minutes.

3.4.4 Preincubation of recombinant CYP2D6 with 1-aminobenzotriazole and quinidine

Previously, 1-aminobenzotriazole was used as a positive control for mechanism-based inactivation of CYP2D6 with human liver microsomes and was shown to inhibit dextromethorphan O-demethylase activity in a time-dependent manner (section 3.3.4). Preincubation of recombinant CYP2D6 with 1-aminobenzotriazole and NADPH resulted in a 51.5% decrease in activity compared to the control group preincubated for the same period of time (figure 32). Without preincubation, no inhibition was observed.

Quinidine, which acts as a competitive inhibitor of CYP2D6 was used as a further control to demonstrate that the time-dependent decrease in activity seen with 1-aminobenzotriazole and cimetidine is not a general property of all CYP2D6 inhibitors. Without preincubation, quinidine competitively inhibited dextrophan formation by 58.3% (figure 33). Following 20 minutes of preincubation with the inhibitor and NADPH, an inhibition of 41.2% was observed. The preincubation step, therefore, did not result in a further decrease in activity with a competitive inhibitor. The only experimental conditions under which a significant decrease in activity was demonstrated with preincubation occurred with the known mechanism-based inactivator, 1-aminobenzotriazole.

Figure 32: Mechanism-based inactivation of recombinant CYP2D6 by 1-aminobenzotriazole (ABT). Recombinant CYP2D6 (10 pmol/mL) was preincubated with 4 μ M ABT, or an equal volume of buffer (control), and NADPH (1 mM). At 0 and 15 minutes after initiation of the preincubation reaction, a 50 μ L aliquot was removed and added to a reaction tube containing dextromethorphan (1 μ M) and NADPH (1 mM), and incubated for a further 15 minutes. Each bar represents the mean of four measurements + standard deviation. * significantly different ($p < 0.05$) from preincubated control. There was a significant interaction ($p < 0.05$) between preincubation time and treatment ABT.

Figure 33: Lack of mechanism-based inactivation of recombinant CYP2D6 by quinidine. Recombinant CYP2D6 (10 pmol/mL) was preincubated with 0.03 μM quinidine or an equal volume of buffer (control), and NADPH (1 mM). At 0 and 20 minutes after initiation of the preincubation reaction, a 50 μL aliquot was removed and added to a reaction tube containing dextromethorphan (1 μM) and NADPH (1 mM), and incubated for a further 15 minutes. Each bar is the mean of four measurements + standard deviation. * significantly different ($p < 0.05$) from control, # significantly different ($p < 0.05$) from and quinidine-treated group that was not preincubated.

3.4.5 Effect of preincubation on the K_i of inhibition by cimetidine

After the preincubation conditions for cimetidine had been established, the K_i determination was repeated with the preincubation step included. Initially, this was carried out using the same preincubation method described in the *Materials and Methods* (section 2.2.3). With this method, the enzyme and inhibitor concentrations in the preincubation reaction are diluted four-fold in the final incubation. Dixon plots were constructed using the cimetidine concentration in the final incubation reaction (figure 34) and the K_i was estimated to be $23.6 \pm 7.5 \mu\text{M}$ ($n=3$). However, because the concentrations of cimetidine and CYP2D6 in the preincubation and incubation steps were different, these results are difficult to interpret.

To overcome this issue, the experiment was repeated using a method that did not involve the dilution step, as described in the legend for figure 35. Briefly, recombinant CYP2D6 (2.5 pmol/mL) was preincubated with cimetidine and NADPH in a volume of 190 μL . After 20 minutes, dextromethorphan was added in a volume of 10 μL , to give a final incubation volume of 200 μL . Dixon plots were constructed, this time using the initial cimetidine concentrations, and the K_i was estimated to be $40.5 \pm 3.5 \mu\text{M}$ ($n=3$). A representative Dixon plot is shown in figure 35.

There was no significant difference between the K_i values determined with these two methods and addition of the preincubation step lowered cimetidine's K_i by 2.5- to 4-fold compared to the value obtained without preincubation (table 11). In both cases, a mixed inhibition model was used to estimate the K_i values, but, based on R^2 values, it was difficult to distinguish which inhibition model provided the best fit for the data. When the dilution method was used, the mixed and noncompetitive inhibition models both provided a good fit for the data, with R^2 values of 0.945 and 0.940, respectively. With the second preincubation method, however, the competitive and mixed models provided similar fits to the data with R^2 values of 0.976 and 0.975, respectively. To facilitate comparison of the K_i values determined previously and with the two preincubation methods, the mixed inhibition model was used in both cases.

Figure 34: Inhibition of recombinant CYP2D6 by cimetidine following 20 minutes preincubation using the dilution method. Recombinant CYP2D6 (10 pmol/mL) was preincubated with cimetidine (25 to 200 μ M) and NADPH (1 mM). After 20 minutes, 50 μ L aliquots were removed from the preincubation mixtures and added to reaction tubes containing dextromethorphan and NADPH in incubation buffer in a volume of 150 μ L. In the final incubation, the concentration of cimetidine ranged from 6.25 to 50 μ M, and the CYP2D6 concentration was 2.5 pmol/mL. Data were fit to the mixed inhibition model using the SigmaPlot enzyme kinetics module. Each point represents the mean of two measurements. Panel A: Dixon plot. Panel B: Lineweaver-Burk transformation of the data presented in panel A.

Figure 35: Dixon plot for the inhibition of recombinant CYP2D6 by cimetidine following 20 minutes preincubation. Recombinant CYP2D6 (2.5 pmol/mL) was preincubated with NADPH (1 mM) and cimetidine (6.25 to 50 μM) in a volume of 190 μL . After 20 minutes, 10 μL dextromethorphan was added to each reaction tube and samples were incubated for a further 15 minutes. Data was fit to a mixed inhibition model using the SigmaPlot enzyme kinetics module. Each point represents the mean of duplicate measurements. A: Dixon plot. B: Lineweaver-Burk transformation of the data shown in panel A.

Table 11: Summary of K_i values for the inhibition of dextromethorphan α -demethylase activity of recombinant CYP2D6 by cimetidine. K_i values were determined without a preincubation step and with a 20 minute preincubation step. The preincubation experiments were conducted with or without a dilution step, as described in the legends for figures 34 and 35, respectively. K_i values were estimated using the mixed inhibition model. * significantly different ($p < 0.05$) than the average K_i values determined with preincubation by either method. There was no statistically significant difference between the two groups of K_i values determined with preincubation.

Trial	cimetidine K_i (μ M)		
	Without preincubation	With preincubation	
		with dilution	without dilution
1	117.6	18.9	41.8
2	105.5	32.2	36.5
3	84.3	19.7	43.2
mean	102.5*	23.6	40.5
standard deviation	16.8	7.5	3.5

To further clarify which mode of inhibition was occurring under conditions of preincubation, higher concentrations of dextromethorphan were included in the Dixon plot (figure 36A). Under these conditions, the non-competitive component of mixed inhibition should be more apparent, if this type of inhibition is occurring. However, in this case, both the mixed and competitive models of inhibition fit the data with the same R^2 value (0.902) and the Lineweaver-Burk plots for each of these models are identical (figure 36B).

Figure 36A: Dixon plot for the inhibition of recombinant CYP2D6 by cimetidine following 20 minutes preincubation using saturating substrate concentrations. Recombinant CYP2D6 (2.5 pmol/mL) was preincubated with NADPH (1 mM) and cimetidine (6.25 to 50 μM) in a volume of 190 μL . After 20 minutes, 10 μL dextromethorphan was added to each reaction tube and the samples were incubated for a further 15 minutes. Each point represents the mean of duplicate measurements. Solid lines represent fit of a mixed inhibition model; however, this model does not appear to provide an adequate fit to the data.

Figure 36B: Lineweaver-Burk transformation of the data presented in figure 36A. Data were fit to the mixed (panel A) or competitive (panel B) model of inhibition.

3.4.6 *Effect of quinidine on inactivation of CYP2D6 by cimetidine*

Mechanism-based inactivators require catalytic activation to a reactive species which then binds to and inactivates the target enzyme. Like other enzyme-mediated reactions, this activation step is susceptible to competitive inhibition. To determine whether the time- and NADPH-dependent decrease in dextromethorphan *O*-demethylase activity by cimetidine could be attenuated by a competitive inhibitor of CYP2D6, increasing concentrations of quinidine were included in the preincubation reaction. The concentrations of quinidine used were equivalent to 0.5-, 1-, and 2-times the K_i of inhibition of dextromethorphan *O*-demethylation; a saturating substrate concentration was used in the incubation reaction to ensure that quinidine would not competitively inhibit the formation of dextromorphan.

Without preincubation, there was no significant inhibition caused by either cimetidine or quinidine (figure 37). Preincubation with NADPH alone for 20 minutes reduced the dextromethorphan *O*-demethylase activity to 8.65 nmol/min/nmol P450, a decrease of approximately 50% compared to the control group which was not preincubated. In the presence of 100 μ M cimetidine, preincubation with NADPH lowered the activity to 7.23 nmol/min/nmol P450, a decrease of 16.8%, which was consistent with the effect previously observed with this inhibitor concentration (section 3.4.3). With the addition of increasing amounts of quinidine to the preincubation mixture, the effect of cimetidine was attenuated and, at the two highest concentrations of quinidine, the activity in the cimetidine-treated groups was not significantly different from that of the control group. The effect of preincubation on inactivation of CYP2D6 was blocked by the addition of quinidine to the preincubation reaction.

Figure 37: Effect of quinidine on inactivation of recombinant CYP2D6 by cimetidine. Recombinant CYP2D6 (10 pmol/mL) was preincubated with cimetidine (100 μ M) and varying concentrations of quinidine (0 to 2.6 nM), and NADPH (1 mM) for 0 or 20 minutes. A 50 μ L aliquot was removed and added to a reaction tube containing dextromethorphan and NADPH in a volume of 150 μ L and was incubated for a further 15 minutes. The concentration of dextromethorphan in the final incubation was 20 μ M. Activity in each group was normalized to the activity of the control group which was preincubated with NADPH (1 mM) for 0 or 20 minutes in the absence of cimetidine or quinidine. Data are expressed as the mean of three experiments + standard deviation. * significantly lower ($p < 0.05$) compared to group containing the same concentration of quinidine that was not preincubated. There was no difference in the activity of the groups that were not preincubated.

3.4.7 Determination of the $k_{\text{inactivation}}$ of cimetidine

As a final demonstration of cimetidine's ability to act as a mechanism-based inactivator of recombinant CYP2D6, the rate constant for inactivation (k_{inact}) was estimated. This was achieved by preincubating the recombinant enzyme with increasing concentrations of cimetidine (50 to 400 μM) for 0 to 20 minutes (figure 38A). The observed decrease in activity due to preincubation with cimetidine was time- and concentration-dependent. Based on a Kitz-Wilson transformation of the data (figure 38B), in which the half-life of inactivation at each concentration of cimetidine was plotted against the inverse concentration of cimetidine in the incubation reaction, the k_{inact} , or the rate of maximal inactivation, was estimated to be 0.03 min^{-1} and the time required to inactivate 50% of the enzyme was estimated to be 25.3 minutes. The concentration of cimetidine required for half-maximal inactivation (K_i) was estimated to be 19.2 μM .

3.4.8 Summary of studies with recombinant CYP2D6

Cimetidine acted as a mixed inhibitor of CYP2D6, with a K_i of 102.5 μM , while quinidine was a competitive inhibitor with a K_i of 1.3 nM. Preincubation of recombinant CYP2D6 with cimetidine resulted in an increase in inhibition, which was dependent on both preincubation time and the presence of NADPH. The maximal effect, observed after 20 minutes of preincubation, resulted in a further 18% decrease in activity compared to the group treated with the same concentration cimetidine in the absence of preincubation. The same type of effect was seen when recombinant CYP2D6 was preincubated with 1-aminobenzotriazole and NADPH; in contrast, preincubation with quinidine did not have an effect on inhibition. Preincubation with cimetidine lowered the K_i of the inhibitor by 2.5- to 4-fold, and was characterized by a k_{inact} of 0.03 min^{-1} and a K_i of 19.2 μM . The inclusion of quinidine in the preincubation reaction attenuated the degree of inactivation of CYP2D6 by cimetidine.

Figure 38: Inactivation of recombinant CYP2D6 by cimetidine. Recombinant CYP2D6 (10 pmol/mL) was preincubated with buffer (control) or various concentrations of cimetidine, and NADPH (1 mM). Immediately before initiating the preincubation reaction and 5, 10, and 20 minutes later, 50 μL aliquots of the preincubation mixture were added to reaction tubes containing dextromethorphan (20 μM) and NADPH (1 mM) and were incubated for a further 15 minutes. A: plot of log percent control activity versus preincubation time. Each point represents the mean of two separate experiments in which measurements were made in triplicate. The concentration of cimetidine reported is that in the final incubation. B: Kitz-Wilson plot of half-life of inactivation versus inverse concentration of cimetidine.

Chapter 4

DISCUSSION

In vitro studies have demonstrated that cimetidine is a weak, reversible inhibitor of various P450 enzymes, including CYP2D6. Cimetidine has been implicated in several drug-drug interactions involving known substrates of this enzyme, suggesting that enzyme inhibition may be occurring at the relatively low plasma concentrations of cimetidine seen *in vivo*. It has been previously shown with rat hepatic microsomes that both the potency and selectivity of inhibition by cimetidine can be increased *in vitro* when experimental conditions allowing for mechanism-based inactivation are used. The purpose of the present study was to further characterize the inhibition of CYP2D6 activity by cimetidine and to determine whether mechanism-based inactivation could be observed under the appropriate experimental conditions. To this end, two *in vitro* model systems were used: human liver microsomes and recombinant CYP2D6. The results obtained from these two experimental models, however, were not in agreement. With human liver microsomes, cimetidine was found to act as a competitive inhibitor of dextromethorphan O-demethylase activity, while with the recombinant enzyme, both mixed inhibition and mechanism-based inactivation were observed, depending on the experimental conditions used. There are several possible explanations for this discrepancy, which relate to the intrinsic properties of each of the model systems used, such as their relative rates of CYP2D6 activity and the presence or absence of additional P450 enzymes. The findings from this study suggest that the choice of model system can have a major impact on the types of conclusions drawn from *in vitro* studies.

Using pooled human liver microsomes, the dextromethorphan O-demethylase activity was comparable to that reported by other laboratories using similar experimental conditions. In the present study, the K_m and V_{max} of the reaction were estimated to be 4.1 μM and 0.82 nmol/min/nmol P450 (or 0.31 nmol/min/mg protein), respectively. Previously reported values range from 2.8 μM to 8.3 μM for the K_m , and 0.14 to 0.26 nmol/min/mg protein for the V_{max} (Dayer *et al.* 1989, Kerry *et al.* 1994, Schmider *et al.* 1997, von Moltke *et al.* 1998). This indicates that the microsomes exhibited expected CYP2D6 expression and activity. The effect of quinidine on dextromethorphan O-demethylation was also consistent with that reported by other investigators. The inhibitor was found to act competitively, with a K_i of 53 nM, which is within the range of 28 nM to 400 nM previously reported (Kobayashi *et al.* 1989, Ching *et al.* 1995, Bourrié *et al.* 1996). Inhibition of CYP2D6 by cimetidine has also been investigated, to a lesser extent than quinidine, with human liver microsomes. Using bufuralol hydroxylase activity as a marker of CYP2D6 activity, the K_i of cimetidine was estimated to be 50 μM (Knodell *et al.* 1991) and 55.5 μM (Furuta *et al.* 2001). The former study also found that cimetidine was acting as a mixed inhibitor. Martinez *et al.* (1999) estimated that cimetidine inhibited dextromethorphan O-demethylase activity of human liver microsomes with an IC_{50} of 210 μM . In this experiment, a relatively high concentration of dextromethorphan was used (25 μM , or 2.5-times the K_m), which may explain the high concentration of cimetidine required to inhibit the activity by 50%. Competitive inhibition is dependent on both substrate and inhibitor concentrations, and the degree of observed competitive inhibition is reduced at higher substrate concentrations. Also, at this substrate concentration, there is likely a considerable contribution of other P450 enzymes to the formation of dextromethorphan, which may be refractory to inhibition by cimetidine. In the present study, inhibition of CYP2D6 by cimetidine was found to occur by competitive inhibition, with a K_i of 38 μM . Despite the differences in the probe drugs used, this value is in agreement with the K_i values obtained in the studies reported above. The difference in the mechanism of inhibition may be a result of differences in methodology. The study which found cimetidine acted as a mixed inhibitor used substrate

concentrations which ranged from slightly below the K_m of the probe reaction to 5-times greater than the K_m , whereas the substrate concentrations used here were limited to 0.5- to 2-times the K_m . If the inhibitor is in fact acting by a combination of both competitive and non-competitive mechanisms, this would be more apparent at higher substrate concentrations.

Preincubation of human liver microsomes with cimetidine and NADPH did not affect the potency of the inhibitor, suggesting that cimetidine does not act as a mechanism-based inactivator of CYP2D6. However, an alternative explanation for this lack of observable effect may be due to the use of inappropriate experimental conditions. For example, the preincubation time may not have been long enough to allow for the production of a sufficient amount of the putative inhibitory species to result in a significant, measurable, degree of enzyme inactivation. The preincubation reaction was carried out for a maximum of 120 minutes. At this point, the activity of the control groups had decreased by 25% to 40%, depending on which human liver microsomes were used, and it was not practical to further extend the duration of preincubation for this reason. A significant degree of lipid peroxidation is known to occur during *in vitro* incubations with hepatic microsomes which leads to a loss of enzyme activity and destruction of the P450 heme (Levin *et al.* 1973, Renton *et al.* 1976). The NADPH concentration-dependent formation of superoxide and hydrogen peroxide, which can be converted to the lipid peroxidation-inducing hydroxy radical in the presence of iron, has been implicated (Bondy and Naderi 1994, Rashba-Step and Cederbaum 1994).

Another possible explanation for the lack of effect may relate to the inability to distinguish competitive inhibition from mechanism-based inactivation. If there is a significant overlap in the concentration of inhibitor that produces reversible and mechanism-based inhibition, it is possible that the reversible inhibition may be sufficient to mask the effect of preincubation on a further decrease in enzyme activity. To minimize the contribution of competitive inhibition, a saturating substrate concentration can be used in the inhibition studies. Unfortunately, this methodology is problematic when using human liver microsomes because at concentrations above two times the K_m ,

dextromethorphan loses its selectivity as a probe of CYP2D6 activity (von Moltke *et al.* 1998). However, the way in which the present studies were performed minimized the concentration of cimetidine present in the final incubation reaction. The transfer of an aliquot of the preincubation mixture into a separate reaction tube for the incubation reaction resulted in a four-fold dilution of the cimetidine concentration. As a result, the decrease in activity attributable to competitive inhibition was only 10% at the highest concentration of cimetidine used. Therefore, unless preincubation was having only a very small effect on the potency of cimetidine as an inhibitor of dextromethorphan O-demethylase activity, it is unlikely that the effect of competitive inhibition would be sufficient to obscure any further inactivation of the enzyme. When a saturating concentration of dextromethorphan was used, preincubation still had no effect on inhibition by cimetidine. It is not possible to conclude whether this observation is due to the absence of mechanism-based inactivation or due to the loss of specificity of dextromethorphan formation as a marker of CYP2D6 activity at this higher substrate concentration.

The experimental method used for the preincubation studies may also have contributed to the lack of effect of preincubation on inhibition by cimetidine by reducing the free concentration of inhibitor due to binding to the microsomal protein. The concentration of protein in the preincubation reaction was 2 mg/mL, four times higher than that used in the competitive inhibition studies. The inhibitor may have been binding non-specifically to the protein, or to specific P450 enzymes other than CYP2D6, thus reducing the amount of cimetidine available to inactivate CYP2D6. Spectral binding studies have shown that cimetidine does bind to at least two rat microsomal P450 enzymes (Rendic *et al.* 1979), and *in vitro* studies have demonstrated that activities of CYPs 1A2 and 3A can be inhibited by cimetidine, therefore the inhibitor may also be binding to these enzymes.

Human liver microsomes provide a good *in vitro* model for hepatic metabolism because all P450 enzymes and accessory proteins are present in their appropriate proportions; however, this can be problematic when trying to isolate the effect of an inhibitor on a single enzyme. Due to overlaps in

substrate specificity, a loss in activity in one enzyme can be compensated for by other enzymes which are capable of mediating the same metabolic pathway. Although CYP2D6 displays the highest affinity for dextromethorphan O-demethylase activity, this pathway of metabolism has a limited capacity due to the low expression of CYP2D6 in the human liver compared to the other enzymes involved in the metabolism of dextromethorphan. Specific inhibition of CYP2D6 may therefore be difficult to observe using this marker activity because other enzymes may compensate for a loss of activity and prevent a further decrease in dextromethorphan formation.

It could be argued that the lack of effect after preincubation of human liver microsomes with cimetidine was due to a flaw in the methodology used for these experiments. However, two positive controls for mechanism-based inactivation, triacetyloleandomycin and 1-aminobenzotriazole, both demonstrated that this type of inhibition could be observed under the same experimental conditions used for the cimetidine preincubation experiments. On the other hand, these two inhibitors are relatively potent, and the experimental conditions required to observe the same type of inhibition with a potentially less potent inhibitor, such as cimetidine, may be quite different. The lack of effect with 120 minutes of preincubation suggests that, if cimetidine is capable of acting as a mechanism-based inactivator, the turnover of the inhibitor to the inactivating species is very slow. This is consistent with *in vivo* observations, where the degree of P450-mediated biotransformation of cimetidine is a relatively minor pathway of metabolism (Mitchell *et al.* 1982) and a period of several days is required to achieve maximal inhibition following administration of cimetidine to patients (Reimann *et al.* 1981). Therefore, it is possible that this *in vitro* system is not capable of supporting the required enzyme activity for the duration of time needed to produce a sufficient amount of the inactivator to cause significant loss of enzyme activity.

Incongruously, preincubation with cimetidine for long periods of time with microsomes from individuals with high CYP2D6 activity appeared to cause an increase in activity compared to the appropriate controls. After preincubation for 120 minutes, the activity in the cimetidine-treated groups

was 25% to 43% higher than the control groups (figures 19 and 20). Although this could be interpreted as enzyme activation, the absolute activity in the cimetidine-treated groups is not actually increasing with time. Instead, this apparent increase in activity was due to a greater decrease in the control activity with than that of the cimetidine-treated groups. The failure of the enzyme activity to decrease in the inhibitor-treated group could have occurred because cimetidine was having a stabilizing effect on CYP2D6 or could have been due to consumption of excess NADPH by metabolism of cimetidine, therefore minimizing the effect of NADPH on lipid peroxidation. This last point cannot be supported based on the current experimental results as the metabolism of cimetidine was not monitored. However, it is unlikely to be a factor as P450-mediated metabolism is only a minor elimination pathway for cimetidine and the amount of NADPH consumed during this process would be very small. It is important to note that these experiments were conducted only once with microsomes from each individual, and this effect needs to be further characterized before conclusions can be drawn.

When higher substrate concentrations were used, the formation of the second metabolite, 3-methoxymorphinan, could be measured. This metabolite has been used as a probe of CYP3A activity, although its lack of selectivity has been documented (Wang and Undakat 1999). It has been suggested that CYP3A may be responsible for a maximum of only 50% of dextromethorphan *N*-demethylase activity and maximal inhibition by triacetyloleandomycin was 50% using concentrations that inhibit testosterone 6 β -hydroxylase activity almost completely (Schmider *et al.* 1997). When triacetyloleandomycin was used as a positive control for mechanism-based inhibition in this study, an effect of similar magnitude was seen. Although preincubation with cimetidine appeared to have a slight, but significant, effect on inactivation of dextromethorphan *N*-demethylase activity, it is difficult to conclude whether cimetidine is in fact acting as a mechanism-based inactivator of CYP3A due to both the magnitude of the effect and the lack of specificity of the probe substrate. If this effect is to be

further investigated, a more specific probe of CYP3A, such as testosterone 6 β -hydroxylase activity, should be used, in order to be more confident that the monitored activity is primarily due to CYP3A.

The estimated K_m for the recombinant enzyme was similar to that obtained with human liver microsomes, but the V_{max} was much higher, leading to a greater than sixty-fold increase in the intrinsic clearance of dextromethorphan by the recombinant enzyme compared to hepatic microsomes. This large difference in the maximal rate of the reaction between the two model systems is expected for a number of reasons. First, with human liver microsomes, the amount of CYP2D6 present represents approximately 2% of the total P450 content (Smith *et al.* 1998); when the recombinant enzyme is used, however, the enzyme concentration is much higher than would normally be found in the human liver. Also, in the recombinant system, the ratio of the accessory protein NADPH P450-reductase to CYP2D6 is different than would be found under physiological conditions, and this can affect the activity of a given P450 enzyme in different preparations (Rommel and Burchell 1993). In recombinant systems the proportion of the reductase can be increased through co-expression of this protein with the P450 and this has been found to result in an elevation of the maximal activity of the P450 enzyme (Nakajima *et al.* 2002, Yamazaki *et al.* 2002). Finally, differences in the osmolarity of the buffer systems used with the two different enzyme preparations can affect maximal activity (Rommel and Burchell 1993). The activity of enzymes produced using different expression systems can also vary considerably. Recombinant P450 enzymes expressed using human lymphoblast cells generally have activities which are more similar to human liver microsomal enzyme activities than are the baculovirus-insect cell-expressed enzymes used in the present study (Gonzalez and Korzekwa 1995).

As was observed with the human liver microsomes, quinidine acted as a competitive inhibitor of CYP2D6. With the recombinant enzyme, however, the K_i was an order of magnitude lower, perhaps reflecting the absence of contribution of other enzymes to dextromethorphan O-demethylase

activity which are refractory to inhibition by quinidine. Cimetidine, in contrast, demonstrated mixed inhibition of recombinant CYP2D6.

Preincubation of the recombinant enzyme with cimetidine resulted in a consistent slight, but significant, increase in inhibition, the magnitude of which depended on the concentration of both the substrate and inhibitor used. A preincubation period of 20 minutes resulted in a further 11% to 36% decrease in dextromethorphan *O*-demethylase activity. The greatest effect with preincubation was observed when 50 μ M cimetidine and 1 μ M dextromethorphan were used in the preincubation and incubation reactions, respectively. At this substrate concentration, which approximated the K_m of the probe reaction, a small degree of competitive inhibition by cimetidine was apparent in the absence of preincubation. The final drop in activity of 36% was, therefore, due to a combination of both reversible inhibition and mechanism-based inactivation. By using a higher substrate concentration, the contribution of reversible inhibition was eliminated. When a saturating substrate concentration was used, the resulting 18% or 11% decrease in activity following preincubation with 100 μ M or 50 μ M cimetidine, respectively, for 20 minutes can be attributed to mechanism-based inactivation of CYP2D6. Regardless of the substrate or inhibitor concentrations used, this further decrease in activity was only seen when the enzyme was preincubated in the presence of NADPH and was dependent on preincubation time, supporting the role of a reactive metabolite or intermediate species in the inactivation of CYP2D6. The effect of preincubation on inhibition by cimetidine was also consistent with that seen with 1-aminobenzotriazole, a known mechanism-based inactivator. In contrast, inhibition of CYP2D6 by quinidine, a known reversible inhibitor, was not affected by preincubation.

In addition to being time- and NADPH-dependent, cimetidine demonstrated several other properties of mechanism-based inactivation, including substrate protection. During the preincubation reaction, a mechanism-based inactivator is catalytically converted to the reactive species that acts as the enzyme inactivator and, like all enzymatic reactions, this process is susceptible to competitive inhibition. In the present study, quinidine was used as the 'substrate', or competitive inhibitor of

CYP2D6, to reduce the formation of the inactivator species. By increasing the concentration of quinidine from 0.5- to 2-times the K_i value for this inhibitor, the effect of preincubation with cimetidine was attenuated in a dose-dependent manner. The inactivation of CYP2D6 was reduced from 17% in the absence of quinidine, to 12% in the presence of the lowest concentration of quinidine. At the two highest concentrations of quinidine, the effect of preincubation on inactivation of CYP2D6 was completely abolished. This further supports the hypothesis that cimetidine is acting as a mechanism-based inactivator of CYP2D6, using this model system.

Preincubation of the recombinant enzyme with cimetidine increased the potency of the inhibitor, as was reflected in a 2.5- to 4-fold decrease in K_i . Without preincubation, cimetidine appeared to be acting as a mixed inhibitor of recombinant CYP2D6; following preincubation, it was more difficult to determine which model of inhibition provided the best fit for the data. When lower dextromethorphan concentrations were used (approximately 0.5-, 1-, and 2-times the K_m), there was little difference between the fit of the competitive, mixed and non-competitive models. The effect of a mechanism-based inactivator on enzyme activity would be expected to be similar to that of non-competitive, reversible inhibitors, which cause a decrease in the maximal rate of the inhibited reaction. Following binding of the inactivator species, the affected enzyme is rendered catalytically inactive and the total number of enzyme molecules available to catalyze the reaction of interest is decreased, thus resulting in a lower V_{max} . The enzyme molecules which are not inactivated still function normally so that the binding of the substrate is not affected and the K_m is unaltered. In this case, the decrease in enzyme activity should be apparent even at saturating substrate concentrations. When the K_i determination was repeated under these conditions, the kinetics module was unable to find an appropriate fit for the data, as was demonstrated in figure 36A. This suggests that the effect of cimetidine on recombinant CYP2D6 is too complex to be fit to a simple model of inhibition. This could be due to an overlap between the concentrations of cimetidine which cause competitive

inhibition and mechanism-based inactivation. Activity of CYP2D6 could, therefore, be affected by more than one inhibitory mechanism simultaneously.

Inhibition and inactivation, however, are two distinct process, and a K_i value does not provide the best description of the latter process. The effectiveness of a mechanism-based inactivator is more appropriately defined by the maximal rate of formation of the inactivator species, or k_{inact} , and the concentration of inhibitor required to produce a half-maximal rate of inactivation (K_I). The k_{inact} and K_I of cimetidine were estimated to be 0.03 min^{-1} and $19.2 \text{ }\mu\text{M}$, respectively. Compared to other well characterized mechanism-based inactivators, cimetidine is relatively weak. For example, furafylline, a selective and potent mechanism-based inactivator of CYP1A2 (Newton *et al.* 1995), is characterized by a k_{inact} of 0.3 min^{-1} and a K_I of $18 \text{ }\mu\text{M}$ (Clarke *et al.* 1994).

The modest effect of preincubation on dextromethorphan O-demethylase activity can be partially explained by its long half-life of inactivation. From the Kitz-Wilson plot in figure 38, the time required to inactivate 50% of the enzyme is approximately 25 minutes, which is longer than the preincubation periods used in these studies. As was seen with human liver microsomes, control activity decreased with increasing preincubation times. With the recombinant enzyme, this effect was much more pronounced and occurred more rapidly: by 20 minutes, approximately 50% of control activity was lost. For this reason, the preincubation period could not be prolonged. Presumably, if the preincubation period could be extended, a greater effect would be observed.

The most striking difference between the two model systems used in the present study was the elevated catalytic efficiency of the recombinant enzyme compared to human liver microsomes for dextromethorphan O-demethylation. This observation may partially explain the discrepancy between the mechanisms of inhibition of cimetidine observed in the two model systems. In order to act as a mechanism-based inactivator, a compound must be catalytically converted to a reactive species by the enzyme that is subsequently inactivated. Using the recombinant enzyme, the present study provided

several key pieces of evidence to indicate that cimetidine was converted into a reactive species which, in turn, was acting as a mechanism-based inactivator of CYP2D6: preincubation with the inhibitor and NADPH for a finite period of time resulted in a decrease in enzyme activity that was apparent even in the presence of high substrate concentrations capable of blocking any competitive inhibition; competitive inhibition of CYP2D6 during the preincubation reaction blocked the effect of preincubation on inactivation by cimetidine; and the rate of inactivation of CYP2D6 could be estimated. This final piece of information indicated that a preincubation period of approximately 25 minutes was required to inactivate 50% of the enzyme. Thus, the preincubation conditions used were insufficient to observe the maximal effect of cimetidine on recombinant CYP2D6 activity, despite the relatively high rate of turnover of the recombinant enzyme. It is not surprising that the same effect was not seen when cimetidine was preincubated with human liver microsomes. In this case, the relative intrinsic clearance of dextromethorphan was approximately 60-fold smaller than that seen with the recombinant enzyme; therefore, the rate of turnover of cimetidine would also be expected to be considerably slower. Given that optimal preincubation conditions could not be achieved using the recombinant system, it is even less likely that these conditions could be established with the microsomal model.

The second major difference between the two *in vitro* model systems involves the specificity of the probe reaction used to evaluate enzyme activity. In the case of the recombinant enzyme, the formation of dextrorphan is obviously exclusively due to CYP2D6-mediated dextromethorphan O-demethylation. However, with human liver microsomes, several P450 enzymes have been shown to be involved in this reaction. As a result, the measured dextromethorphan O-demethylase activity may reflect the activity of two or more P450 enzymes, with the contribution of each enzyme dependent on the substrate and inhibitor concentrations used. The effect of an inhibitor on a single enzyme is therefore difficult to isolate using human liver microsomes and is dependent on the experimental conditions. The inability to observe the same effect of preincubation with cimetidine on recombinant

and microsomal CYP2D6 activity may have been a result of this. With human liver microsomes, other enzymes may have been compensating for loss in activity of CYP2D6. Additionally, in order to maintain maximal selectivity of the probe reaction for CYP2D6, low substrate concentrations had to be used. However, mechanism-based inactivation is best observed when saturating substrate concentrations are used, as was seen with the recombinant enzyme. The conditions under which the microsomal studies were conducted were a balance between maximizing the specificity of the probe reaction and optimizing the conditions under which mechanism-based inactivation could be observed and the optimal experimental conditions for observing each of these conditions were compromised. As a result, it is unlikely that the effect of preincubation with cimetidine on the activity of the recombinant enzyme could be mimicked using the microsomal system.

The *in vitro* experiments carried out in this study indicate that cimetidine is a weak reversible inhibitor, and potentially a mechanism-based inactivator, of CYP2D6. The results described here are inadequate to fully explain the *in vivo* effect of cimetidine on the clearance of CYP2D6-metabolized drugs noted in a number of clinical studies, based on a maximal plasma concentration of cimetidine of 10 μ M. The concentration of the inhibitor at the active site(s) of the affected enzyme(s), however, is unknown and it is possible that cimetidine may be concentrated within the liver compared to plasma. Furthermore, the clinical observation of inhibited drug metabolism is dependent not only on the concentration of the inhibitor, but also that of the substrate whose biotransformation is impaired. If the substrate is present at a low concentration relative to the K_m of the reaction pathway, it will be more susceptible to inhibition. Further *in vivo* experimentation is therefore required in order to fully understand the mechanism(s) underlying the clinical observations.

Chapter 5

FUTURE STUDIES

5.1 Studies with human liver microsomes

One possible explanation for the lack of effect of preincubation on inhibition by cimetidine is that the contribution of P450 enzymes other than CYP2D6 to the formation of dextrophan is compensating for a loss in activity of CYP2D6. In order to overcome this, the preincubation studies could be repeated with the inclusion of inhibitors (chemical or antibodies) of these other enzymes, such as CYP2C9, which is the major low-affinity enzyme involved in dextrophan formation (von Moltke *et al.* 1998). Assuming that optimal conditions of inhibition could be achieved, resulting in complete inhibition of these other enzymes, the observed dextromethorphan O-demethylase activity should be exclusively due to CYP2D6. Also, these experimental conditions would allow the use of higher substrate concentrations, thus isolating the effect of enzyme inactivation from that of competitive inhibition.

Alternatively, a different substrate could be used to measure CYP2D6 activity. For example, the clearance of both desipramine and nortriptyline, two CYP2D6 substrates, has been shown to be affected by cimetidine *in vivo* (Miller *et al.* 1983, Steiner and Spina 1987). Although dextromethorphan was initially selected as the probe substrate in this study due to its relatively high selectivity for CYP2D6, perhaps using a substrate with which cimetidine had already been demonstrated to interact would have been a more appropriate choice.

In the present study, there was some evidence to suggest that CYP3A may be inhibited by cimetidine. Cimetidine has been shown to inhibit CYP3A4 *in vitro* (Martinez *et al.* 1999), but again, the

effect of preincubation has not been studied. In order to more fully investigate this, a more selective probe of CYP3A activity should be used. The 6 β -hydroxylation of testosterone has been demonstrated to be a selective marker of CYP3A activity. The same types of experiments conducted in this study, including determination of the K_i of cimetidine and the effect of preincubation on this value, could be repeated for this activity, using both human liver microsomes and the recombinant enzyme. These experiments would also provide information on the specificity of cimetidine as a mechanism-based inactivator.

5.2 Studies with recombinant CYP2D6

Although there were various indications that cimetidine was acting as a mechanism-based inactivator of recombinant CYP2D6, the effect of preincubation on the observed model of inhibition was inconsistent with this type of inhibition. With preincubation, one would expect a shift from competitive to non-competitive inhibition; however, with cimetidine, the kinetics modelling software had difficulty fitting any model to the data, but the competitive and mixed inhibition models were found to fit better than the non-competitive model. It would be interesting to determine the effect of preincubation on the observed model of inhibition of quinidine. Preincubation was shown to have no effect on the potency of this inhibitor, therefore, after preincubation, the competitive model should still provide the best fit. Also, the same comparison could be made with 1-aminobenzotriazole, which demonstrated mechanism-based inactivation of CYP2D6. In this case, non-competitive inhibition should be observed.

The results from the studies described in this thesis suggest that the experimental conditions were not optimal for observing mechanism-based inactivation using the recombinant model. Based on the estimated half-life of inactivation of 25.3 minutes, the maximal preincubation time used did not cover even one half-life. However, due to the instability of the recombinant enzyme, the preincubation period cannot be prolonged using the experimental conditions described in this study. However, it has

been shown that the use of antioxidants, such as α -methyldopa, in the preincubation reaction results in an attenuation of the rapid loss in enzyme activity with increasing preincubation times (Veres *et al.* 2001). Although this study was conducted using rat hepatic microsomes, the same type of effect would be expected with recombinant enzymes. Before including an additional compound in the incubation reaction, a series of preliminary experiments would have to be conducted to ensure that the activity of the enzyme was not affected.

If the conditions under which mechanism-based inactivation occurs could be optimized through the use of an antioxidant, it would then be worthwhile to repeat the determination of the k_{inact} , because use of a longer preincubation time would presumably provide a more accurate prediction of this value. Furthermore, the other characteristics of a mechanism-based inactivator that were not addressed in this study could be investigated. The major properties that were not demonstrated include the irreversible nature of the inactivation, the type of complex formed between the reactive species and the enzyme, and inactivation of the enzyme prior to the release of the reactive species from the active site. The first point can be investigated by treating the enzyme with potassium ferricyanide or by determining whether dialysis of the complexed enzyme restores activity. This would also provide evidence as to whether the reactive species covalently or tightly binds to the enzyme. If activity can be restored following treatment with either of the two methods described above, this would suggest that a metabolite-intermediate complex is being formed, while a lack of effect with these agents would indicate covalent modification of the enzyme. The formation of a metabolite-intermediate complex could also be monitored spectrophotometrically as a time-dependent increase in a peak near 420 nm. To evaluate whether the reactive species inactivates the enzyme before being released from the active site, trapping agents such as glutathione or N-acetylcysteine can be used in the preincubation reaction. These compounds can effectively inactivate reactive electrophilic species, and would therefore be able to decrease the effect of preincubation on enzyme inactivation in a concentration-dependent manner if the reactive species were released before binding to the enzyme.

5.3 Studies with recombinant CYP2D6*10

Finally, to address the initial hypothesis presented in section 1.6 regarding the susceptibility of CYP2D6*10 compared to the wild-type enzyme, the experiments conducted with recombinant CYP2D6 presented in this thesis could be repeated using the variant enzyme. Briefly, the kinetic constants of dextromethorphan *O*-demethylation would have to be determined to allow for the rational selection of substrate concentrations for subsequent experiments. The effect of both quinidine and cimetidine on the activity of CYP2D6*10 could then be determined and estimates of the K_i values for each of these inhibitors could be obtained and compared to the K_i values already determined for the wild-type enzyme. The effect of preincubation could then be investigated to determine whether the mechanism-based inactivation observed with the wild-type enzyme could also be observed with CYP2D6*10. Under the present experimental conditions, however, it is unlikely that such inactivation would be observed with the CYP2D6*10 variant. The rate of turnover of the variant enzyme is lower than that of the wild-type enzyme used in the present study and, given the small decrease in activity seen following preincubation with the wild-type enzyme, being able to reproducibly measure the even smaller decrease expected with CYP2D6*10 would be difficult. The use of appropriate controls in the preincubation experiments, such as 1-aminobenzotriazole and quinidine, would be important in such a situation.

Chapter 6

SUMMARY & CONCLUSIONS

- 1. Under the experimental conditions used, cimetidine acted as a competitive inhibitor, but not a mechanism-based inactivator, of dextromethorphan *O*-demethylation in human liver microsomes.**

The estimated K_i for competitive inhibition was higher than the peak plasma concentration of cimetidine observed following clinical administration of the drug. Therefore, competitive inhibition alone cannot explain the drug-drug interactions observed with cimetidine and CYP2D6 substrates. It is possible that the appropriate conditions could not be achieved to observed mechanism-based inhibition in these hepatic microsome samples.

- 2. Using recombinant CYP2D6, cimetidine was shown to act as both a mixed inhibitor and a mechanism-based inactivator.**

Following preincubation of the recombinant enzyme with cimetidine and NADPH, the potency of the inhibitor was increased. This effect was relatively weak, resulting in a further 15% loss of activity or a 2.5- to 4-fold decrease in the K_i , but could potentially be increased if the preincubation period could be extended beyond 20 minutes.

- 3. The type of inhibition seen is dependent on the model system and experimental conditions used.**

The large difference in absolute CYP2D6 activity between the human liver microsomes and recombinant system may explain the differences in the observed mechanisms of inhibition of

cimetidine. The presence or absence of a preincubation step and the concentration of substrate used can also affect the observed inhibition. Based on the present study, an appropriate *in vitro* model was not found to explain the effect of cimetidine on CYP2D6 activity *in vivo*.

Chapter 7

REFERENCES

-
- Abernethy DR, Greenblatt DJ, Shader RI. 1984. Imipramine-cimetidine interaction: impairment of clearance and enhanced bioavailability. *Journal of Pharmacology and Experimental Therapeutics* 229:702-705.
- Amsterdam JD, Brunswick DJ, Potter L, Kaplan MJ. 1984. Cimetidine-induced alterations in desipramine plasma concentration. *Psychopharmacology* 83:373-375.
- Balani SK, Zhu T, Yang TJ, Liu Z, He B, Lee FW. 2002. Effective dosing regimen of 1-aminobenzotriazole for inhibition of antipyrine clearance in rats, dogs, and monkeys. *Drug Metabolism and Disposition* 30:1059-1062.
- Bavin PMG, Post A, Zarembo JE. 1984. Cimetidine. *Analytical Profiles of Drug Substances* 13:127-183.
- Benedetti MS, Bani M. 1998. Design of *in vitro* studies to predict *in vivo* inhibitory drug-drug interactions. *Pharmacological Research* 38:81-88.
- Bodemar G, Norlander B, Walan A. 1981. Pharmacokinetics of cimetidine after single doses and during continuous treatment. *Clinical Pharmacokinetics* 6:306-315.
- Bondy SC, Naderi S. 1994. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochemical Pharmacology* 48:155-159.
- Brosen K, Gram LF. 1989. Clinical significance of the sparteine/debrisoquine oxidation polymorphism. *European Journal of Clinical Pharmacology* 36:537-547.
- Bourri M, Meunier V, Berger Y, Fabre G. 1996. Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics* 277:321-332.
- Brogden RN, Heel RC, Speight TM, Avery GS. 1978. Cimetidine: a review of its pharmacological properties and therapeutic efficacy in peptic ulcer disease. *Drugs* 15:93-131.
- Busby WF, Ackermann JM, Crespi CL. 1998. Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on *in vitro* activities of cDNA-expressed human cytochromes P450. *Drug Metabolism and Disposition* 27:246-249.

- Capon DA, Bochner F, Kerry N, Mikus G, Danz C, Somogyi AA. 1996. The influence of CYP2D6 polymorphism and quinidine on the disposition and antitussive effect of dextromethorphan in humans. *Clinical Pharmacology and Therapeutics* 60:295-306.
- Causon R. 1997. Validation of chromatographic methods in biomedical analysis: viewpoint and discussion. *Journal of Chromatography B* 689:175-180.
- Chang T, Levine M, Bandiera SM, Bellward GD. 1992A. Selective inhibition of rat hepatic microsomal cytochrome P450 I. Effect of the *in vivo* administration of cimetidine. *Journal of Pharmacology and Experimental Therapeutics* 260:1441-1449.
- Chang T, Levine M, Bellward GD. 1992B. Selective inhibition of rat hepatic microsomal cytochrome P450 II. Effect of the *in vitro* administration of cimetidine. *Journal of Pharmacology and Experimental Therapeutics* 260:1450-1455.
- Chang TKH, Gonzalez FJ, Waxman DJ. 1994. Evaluation of triacetyloleandomycin, α -naphthaflavone, and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics* 311:437-442.
- Ching MA, Blake CK, Ghabrial H, Ellis SW, Lennard MS, Tucker GT, Smallwood RA. 1995. Potent inhibition of yeast-expressed CYP2D6 by dihydroquinidine, quinidine, and its metabolites. *Biochemical Pharmacology* 50:833-837.
- Cholerton S, Daly AK, Idle JR. 1992. The role of individual human cytochromes P450 in drug metabolism and clinical response. *Trends in Pharmacological Science* 13:434-439.
- Clarke SE, Ayrton AD, Chenery RJ. 1994. Characterization of the inhibition of P4501A2 by furafylline. *Xenobiotica* 24:517-526.
- Colby HD, Abbott B, Cachovic M, Debolt KM, Mico BA. 1995. Inactivation of adrenal cytochromes P450 by 1-aminobenzotriazole. *Biochemical Pharmacology* 49:1057-1062.
- Copeland RA. 1996. Enzymes: A practical introduction to structure, mechanism, and data analysis. VCH Publishers Inc, New York.
- Crespi CL. 1995. Xenobiotic-metabolizing human cells as tools for pharmacological and toxicological research. *Advances in Drug Research* 26:179-235.
- Crespi CL, Penman BW. 1997. Use of cDNA-expressed human cytochrome P450 enzymes to study potential drug-drug interactions. *Advances Pharmacology* 43:171-188.
- Crespi CL, Miller VP. 1999. The use of heterologously expressed drug metabolizing enzymes - state of the art and prospects for the future. *Pharmacology and Therapeutics* 84:121-131.
- Dalen P, Dahl ML, Ruiz MLB, Nordin J, Bertilsson L. 1998. 10-hydroxylation of nortriptyline in white persons with 0, 1, 2, 3, and 13 functional CYP2D6 genes. *Clinical Pharmacology and Therapeutics* 63:444-452.
- Dayer P, Leemann T, Striberni R. 1989. Dextromethorphan O-demethylation in liver microsomes as a prototype reaction to monitor cytochrome P-450 db₁ activity. *Clinical Pharmacology and Therapeutics* 45:34-40.

- Distlerath LM, Reilly PEB, Martin MV, Davis GG, Wilkinson GR. 1985. Purification and characterization of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin O-demethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. *Journal of Biological Chemistry* 260:9057-9067.
- Ducharme J, Abdullah S, Wainer IW. 1996. Dextromethorphan as an *in vivo* probe for the simultaneous determination of CYP2D6 and CYP3A activity. *Journal of Chromatography B* 678:113-128.
- Ellis SW, Hayhurst GP, Smith G, Lightfoot T, Wong MMS. 1995. Evidence that aspartic acid 301 is a critical substrate-contact residue in the active site of cytochrome P450 2D6. *Journal of Biological Chemistry* 270:29055-29058.
- Feldman M, Burton ME. 1990. Histamine₂-receptor antagonists: standard therapy for acid-peptic diseases (first of two parts). *The New England Journal of Medicine* 323:1672-1680.
- Fukada T, Yamamoto I, Nishida Y, Zhou Q, Ohno M. 1999. Effect of the CYP2D6*10 genotype on venlafaxine pharmacokinetics in healthy adult volunteers. *British Journal of Clinical Pharmacology* 47:450-453.
- Fukuda T, Nishida Y, Imaoka S, Hiroi T, Naihara M. 2000. The decreased *in vivo* clearance of CYP2D6 substrates by CYP2D6*10 might be caused not only by the low-expression but also by low affinity of CYP2D6. *Archives of Biochemistry and Biophysics* 380:303-308.
- Furuta S, Kamada E, Suzuki T, Sugimoto T, Kawabata Y, Shinozaki Y, Sano H. 2001. Inhibition of drug metabolism in human liver microsomes by nizatidine, cimetidine and omeprazole. *Xenobiotica* 31:1-10.
- Garcia-Barcelo M, Chow LY, Chiu HFK, Wing YK, Lee DTS. 2000. Genetic analysis of the CYP2D6 locus in a Hong Kong Chinese population. *Clinical Chemistry* 46:18-23.
- Gonzalez FJ. 1989. The molecular biology of cytochrome P450s. *Pharmacological Reviews* 40:243-288.
- Gonzalez FJ, Korzekwa KR. 1995. Cytochromes P450 expression systems. *Annual Reviews of Pharmacology and Toxicology* 35:369-390.
- Gonzalez FJ, Nebert DW. 1990. Evolution of the P450 superfamily. *Trends in Genetics* 6:182-186.
- Gorski JC, Jones DR, Wrighton SA, Hall SD. 1994. Characterization of dextromethorphan N-demethylation by human liver microsomes. *Biochemical Pharmacology* 48:173-182.
- Griese EU, Zanger UM, Brudermanns U, Gaedigk A, Mikus G. 1998. Assessment of the predictive power of genotypes for the *in vivo* catalytic function of CYP2D6 in a German population. *Pharmacogenetics* 8:15-26.
- Guengerich FP. 2001. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicology. *Chemical Research in Toxicology* 14:611-650.
- Guengerich FP, Muller-Enoch D, Blair IA. 1986. Oxidation of quinidine by human liver cytochrome P450. *Molecular Pharmacology* 30:287-295.
- Halpert JR. 1995. Structural basis of selective cytochrome P450 inhibition. *Annual Review of Pharmacology and Toxicology* 35:29-53.

- Halpert JR, Guengerich FP, Bend JR, Correia MA. 1994. Selective inhibition of cytochromes P450. *Toxicology and Applied Pharmacology* 125:163-175.
- Hasler JA, Estabrook R, Murray M, Pikuleva I, Waterman M. 1999. Human cytochromes P450. *Molecular Aspects of Medicine* 20:1-137.
- Henhauer SA, Hollister LE. 1984. Cimetidine interaction with imipramine and nortriptyline. *Clinical Pharmacology and Therapeutics* 35:183-187.
- Ingelman-Sundberg M, Oscarson M, McLellan RA. 1999. Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. *Trends in Pharmacological Science* 20:342-349.
- Ingelman-Sundberg M, Evans WE. 2001. Unravelling the functional genomics of the human CYP2D6 gene locus. *Pharmacogenetics* 11:553-554.
- Ito K, Iwatsubo T, Kananitsu S, Nakajima Y, Sugiyama Y. 1998. Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annual Reviews of Pharmacology and Toxicology* 38:461-499.
- Johansson I, Oscarson M, Yue QY, Bertilsson L, Sjoqvist F. 1994. Genetic analysis of the Chinese cytochrome P450 locus: characterization of variant CYP2D6 genes present in subjects with diminished capacity for debrisoquine hydroxylation. *Molecular Pharmacology* 46:452-459.
- Jones DR, Gorski JC, Hamman MA, Hall SD. 1996. Quantification of dextromethorphan and metabolites: a dual phenotypic marker for cytochrome P450 3A4/5 and 2D6 activity. *Journal of Chromatography B* 678:105-111.
- Kalow W. 1968. Pharmacogenetics in animals and man. *Annals of the NY Academy of Science* 151:694-698.
- Kalow W. 1971. Topics in pharmacogenetics. *Annals of the NY Academy of Science* 179:654-659.
- Karnes HT, Shiu G, Shah VP. 1991. Validation of bioanalytical methods. *Pharmaceutical Research* 8:421-426.
- Kerry NL, Somogyi AA, Bochner F, Mikus G. 1994. The role of CYP2D6 in primary and secondary oxidative metabolism of dextromethorphan: *in vitro* studies using human liver microsomes. *British Journal of Clinical Pharmacology* 38:243-248.
- Kimura S, Umeno M, Skoda RC, Meyer UA, Gonzalez FJ. 1989. The human debrisoquine 4-hydroxylase (CYP2D) gene locus: sequence and identification of the polymorphic CYP2D6 gene, a related gene, and a pseudogene. *American Journal of Human Genetics* 45:889-904.
- Knodell RG, Browne DG, Gwozdz GP, Brian WR, Guengerich FP. 1991. Differential inhibition of individual human liver cytochromes P450 by cimetidine. *Gastroenterology* 101:1680-1691.
- Kobayashi S, Murray S, Watson D, Sesardic D, Davis DS, Boobis AR. 1989. The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochemical Pharmacology* 38:2795-2799.
- Kubota T, Yamaura Y, Ohkawa N, Hara H, Chiba K. 2000. Frequencies of CYP2D6 mutant alleles in a normal Japanese population and metabolic activity of dextromethorphan O-demethylation in different CYP2D6 genotypes. *British Journal of Clinical Pharmacology* 50:31-34.

- Lam YWF, Rodriguez SY. 1993. High-performance liquid chromatography determination of dextromethorphan and dextrorphan for oxidation phenotyping by fluorescence and ultraviolet detection. *Therapeutic Drug Monitoring* 15:300-304.
- Levin W, Lu AYH, Jacobson M, Kuntzman R. 1973. Lipid peroxidation and the degradation of cytochrome P450 heme. *Archives of Biochemistry and Biophysics* 158:842-852.
- Levine M, Bellward GD. 1995. Effect of cimetidine on hepatic cytochrome P450: evidence for formation of a metabolite-intermediate complex. *Drug Metabolism and Disposition* 23:1407-1411.
- Levine M, Law EYW, Bandiera SM, Chang TKH, Bellward GD. 1998. *In vivo* cimetidine inhibits hepatic CYP2C6 and CYP2C11 but not CYP1A1 in adult male rats. *Journal of Pharmacology and Experimental Therapeutics* 284:493-499.
- Lewis DFW, Dickins M, Eddershaw PJ, Tarbit MH, Goldfarb PS. 1999. Cytochrome P450 substrate specificities, substrate structural templates and enzyme active site geometries. *Drug Metabolism & Drug Interactions* 15:1-49.
- Lin JH, Lu AYH. 1998. Inhibition and induction of cytochrome P450 and the clinical implications. *Clinical Pharmacokinetics* 35:361-390.
- Lindstrom TD, Hanssen BR, Wrighton SA. 1993. Cytochrome P450 complex formation by dirithromycin and other macrolides in rat and human livers. *Antimicrobial Agents and Chemotherapy* 37:265-269.
- Mahgoub A, Idle JR, Dring LG, Lancaster R, Smith RL. 1977. Polymorphic hydroxylation of debrisoquine in man. *Lancet* 2:584-586.
- Marez D, Legrand M, Sabbagh N, Lo Guidice JM, Spire C. 1997. Polymorphism of the cytochrome P450 CYP2D6 gene in a European population: characterization of 48 mutations and 53 alleles, their frequencies and evolution. *Pharmacogenetics* 7:193-202.
- Martinez C, Albet C, Agundez JAG, Herrero E, Carrillo JA, Marquez M, Benitz J, Ortiz JA. 1999. Comparative *in vitro* and *in vivo* inhibition of cytochrome P450 CYP1A2, CYP2D6, and CYP3A by H₂-receptor antagonists. *Clinical Pharmacology and Therapeutics* 65:369-376.
- Miller DD, Macklin M. 1983. Cimetidine-imipramine interaction: a case report. *American Journal of Psychiatry* 140:351-352.
- Miller DD, Sawyer JB, Duffy JP. 1983. Cimetidine's effect on steady-state serum nortriptyline concentrations. *Drug Intelligence and Clinical Pharmacy* 17:904-905.
- Mitchell SC, Idle JR, Smith RL. 1982. The metabolism of [¹⁴C]cimetidine in man. *Xenobiotica* 12:283-292.
- Murray M. 1984. *In vitro* effects of quinoline derivatives on cytochrome P450 and aminopyrine N-demethylase activity in rat hepatic microsomes. *Biochemical Pharmacology* 33:3277-3281.
- Murray M. 1987. Mechanisms of the inhibition of cytochrome P450-mediated drug oxidation by therapeutic agents. *Drug Metabolism Reviews* 18:55-81.
- Murray M. 1997. Drug-mediated inactivation of cytochrome P450. *Clinical and experimental pharmacology and physiology* 24:465-470.

- Nakajima M, Nakamura S, Tokudome S, Shimada N, Yamazaki H. 1999. Azelastine N-demethylation by cytochrome P450 (CYP)3A4, CYP2D6, and CYP1A2 in human liver microsomes: evaluation of approach to predict the contribution of multiple CYPs. *Drug Metabolism and Disposition* 27:1381-1391.
- Nakajima M, Tane K, Nakamura S, Shimada N, Yamazaki H, Yokoi T. 2002. Evaluation of approach to predict the contribution of multiple cytochrome P450s in drug metabolism using relative activity factor: effects of the differences in expression levels of NADPH-dytochromeP450 reductase and cytochrome b₅ in the expression system and the differences in the marker activities. *Journal of Pharmaceutical Sciences* 91:952-963.
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Gotoh O, Coon MJ, Estabrook RW, Gunsalus EC, Nebert DW. 1996. P450 superfamily: update on new sequences, gene mapping accession numbers and nomenclature. *Pharmacogenetics* 6:1-42.
- Newton DJ, Wang RW, Lu AYH. 1995. Evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. *Drug Metabolism and Disposition* 23:154-158.
- Omura T. 1999. Forty years of cytochrome P450. *Biochemical and Biophysical Research Communications* 266: 690-698.
- Ortiz de Montellano PR, Mathews JM. 1981. Autocatalytic alkylation of the cytochrome P450 prosthetic haem group by 1-aminobenzotriazole. *Biochemistry Journal* 195:761-764.
- Ortiz de Motellano PR, Correia MA. 1995. Inhibition of Cytochrome P450 enzymes. *From Ortiz de Monellano, PR (ed). Cytochrome P450: structure, Mechanism, and Biochemistry (Second Edition), Plenum Press, New York.*
- Pelkonen O, Puurunen J. 1980. The effect of cimetidine on *in vitro* and *in vivo* microsomal drug metabolism in the rat. *Biochemical Pharmacology* 29:3075-3080.
- Pessayre D, Larrey D, Vitaux J, Breil P, Belghiti J, Benhamou JP. 1982. Formation of an inactive cytochrome P450 Fe(II)-metabolite complex after administration of troleandomycin in humans. *Biochemical Pharmacology* 31:1699-1704.
- Phillip PA, James CA, Rogers HJ. 1989. The influence of cimetidine on debrisoquine 4-hydroxylation in extensive metabolizers. *European Journal of Clinical Pharmacology* 36:319-321.
- Potkin SG, Shen Y, Pardes H, Phelps BH, Zho D, 1984. Haloperidol concentrations elevated in Chinese pateints. *Psychiatry Research* 12:167-172.
- Puurunen J, Sotaniemi E, Pelkonen O. 1979. Effect of cimetidine on microsomal drug metabolism in man. *European Journal of Clinical Pharmacology* 18:185-187.
- Ramamoorthy Y, Tyndale RF, Sellers EM. 2001. Cytochrome P450 2D6.1 and cytochrome P450 2D6.10 differ in catalytic activity for multiple substrates. *Pharmacogenetics* 11:477-487.
- Rashba-Step J, Cederbaum AI. 1994. Generation of reactive oxygen intermediates by human liver microsomes in the presence of NADPH or NADH. *Molecular Pharmacology* 45:150-157.
- Reimann IW, Klotz U, Siems B, Frolich JC. 1981. Cimetidine increases steady state plasma levels of propranolol. *British Journal of Clinical Pharmacology* 12:785-790.

- Rommel RP, Burchell B. 1993. Validation and use of cloned, expressed human drug-metabolizing enzymes in heterologous cells for analysis of drug metabolism and drug-drug interactions. *Biochemical Pharmacology* 46:559-566.
- Rendic S, Sunjic V, Kajfez F, Ruf HH. 1979. Interactions of cimetidine with liver microsomes. *Xenobiotica* 9:555-564.
- Renton KW, Aranda JV, Eade NR. 1976. NADPH-dependent lipid peroxidation and its effects on aminopyrine N-demethylation in subcellular fractions of human neonatal liver. *Canadian Journal of Physiology and Pharmacology* 56:838-843.
- Rodrigues AD. 1994. Use of in vitro human metabolism studies in drug development. *Biochemical Pharmacology* 48:2147-2156.
- Rudorfer MV, Lane EA, Chang WH, Zhang M, Potter WZ. 1984. Desipramine pharmacokinetics in Chinese and Caucasian volunteers. *British Journal of Clinical Pharmacology* 17:433-440.
- Sachse C, Brockmoller J, Bauer S, Roots I. 1997. Cytochrome P450 2D6 variants in a Caucasian population: allele frequencies and phenotypic consequences. *American Journal of Human Genetics* 60:284-295.
- Sachse C, Brockmoller J, Hildebrand M, Muller K, Roots I. 1998. Correctness of prediction of the CYP2D6 phenotype confirmed by genotyping 47 intermediate and poor metabolizers of debrisoquine. *Pharmacogenetics* 8:181-185.
- Schellens JHM, van der Wart JHF, Brugman M, Breimer DD. 1989. Influence of enzyme induction and inhibition of the oxidation of nifedipine, sparteine, mephenytoin and antipyrine in humans as assessed by a "cocktail" study design. *Journal of Pharmacology and Experimental Therapeutics* 249:638-645.
- Schmider J, Greenblatt DJ, Fogelman SM, von Moltke LL, Shader RI. 1997. Metabolism of dextromethorphan *in vitro*: involvement of cytochromes P450 2D6 and 3A3/4, with a possible role of 2E1. *Biopharmaceutics & Drug Disposition* 18:227-240.
- Serlin MJ, Sibeon RG, Mossman S, Breckenridge AM. 1979. Cimetidine: interactions with oral anticoagulants in man. *Lancet* 2:317-319.
- Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Viswanathan CT, Cook CE, McDowell ED, Pittman KA, Spector S. 1992. Analytical methods validation: bioavailability, bioequivalence, and pharmacokinetic studies. *Pharmaceutical Research* 9:588-592.
- Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. 1994. Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics* 270:414-423.
- Shimada T, Tsumura F, Yamazaki H. 1999. Prediction of human liver microsomal oxidations of 7-ethoxycoumarin and chlorzoxazone with kinetic parameters of recombinant cytochrome P450 enzymes. *Drug Metabolism and Disposition* 27:1274-1280.
- Silverman RB. 1995. Mechanism-based enzyme inactivators. *Methods in Enzymology* 249:240-283.
- Smith G, Stubbins MJ, Harries LW, Wolf CR. 1998. Molecular genetics of the human cytochrome P450 monooxygenase superfamily. *Xenobiotica* 28:1129-1165.

- Somogyi A, Gugler R. 1982. Drug interactions with cimetidine. *Clinical Pharmacokinetics* 7:23-41.
- Somogyi A, Gugler R. 1983. Clinical Pharmacokinetics of cimetidine. *Clinical Pharmacokinetics* 8:463-495.
- Steiner E, Spina E. 1987. Differences in the inhibitory effect of cimetidine on desipramine metabolism between rapid and slow debrisoquin hydroxylators. *Clinical Pharmacology and Therapeutics* 42:278-282.
- Tanaka E. 1999. Update: genetic polymorphism of drug metabolizing enzymes in humans. *Journal of Clinical Pharmacy and Therapeutics* 24:323-329.
- Tateishi T, Chida M, Ariyoshi N, Mizorogi Y, Kamataki T. 1999. Analysis of the *CYP2D6* gene in relation to dextromethorphan O-demethylation capacity in a Japanese population. *Clinical Pharmacology and Therapeutics* 65:570-575.
- Testa B. 1990. Mechanisms of inhibition of xenobiotic-metabolizing enzymes. *Xenobiotica* 20:1129-37.
- Tingle MD, Coleman MD, Park BK. 1991. The effect of preincubation with cimetidine on the N-hydroxylation of dapsone by human liver microsomes. *British Journal of Clinical Pharmacology* 32:120-123.
- Van der Weide J, Steijns LSW. 1999. Cytochrome P450 enzyme system: genetic polymorphisms and impact on clinical pharmacology. *Annals of Clinical Biochemistry* 36:722-729.
- Venkatakrishnan K, von Moltke LL, Court MH, Harmatz JS, Crespi CL. 2000. Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches. *Drug Metabolism and Disposition* 28:1493-1504.
- Veres A, Szakacs T, Vereczkey L. 2001. Effect of α -methyldopa on pentoxifyresorufin O-dealkylation in liver microsomes from rats treated with phenobarbital. *Archives of Biochemistry and Biophysics* 302:59-64.
- Von Moltke LL, Greenblatt DJ, Grassi JM, Granada BW, Venkatakrishnan K, Schmider J, Harmatz JS, Shader RI. 1998. Multiple human cytochromes contribute to biotransformation of dextromethorphan *in vitro*: role of CYP2C9, CYP2C19, CYP2D6, and CYP3A. *Journal of Pharmacy and Pharmacology* 50:997-1004.
- Wang SL, Huang JD, Lai MG, Liu BH, Lai ML. 1993. Molecular basis of genetic variation in debrisoquine hydroxylation in Chinese subjects: polymorphism in RFLP and DNA sequence of *CYP2D6*. *Clinical Pharmacology and Therapeutics* 53:410-418.
- Wang Y, Unadkat JD. 1999. Enzymes in addition to CYP3A4 and 3A5 mediate N-demethylation of dextromethorphan in human liver microsomes. *Biopharmaceutics & Drug Disposition* 20:341-346.
- Yamazaki H, Nakamura M, Komatsu T, Ohyama K, Hatanaka N, Asahi S, Shimada N, Guengerich FP, Shimada T, Nakajima M, Yokoi T. 2002. Roles of NADPH-P450 reductase and apo- and holo-cytochrome b₅ on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. *Protein Expression and Purification* 24:329-337.
- Yoon YR, Cha IJ, Shon JH, Kim KA, Cha YN. 2000. Relationship of paroxetine disposition to metoprolol metabolic ratio and *CYP2D6**10 genotype of Korean subjects. *Clinical Pharmacology Therapeutics* 67:567-576.

Yu A, Haining RL. 2001. Comparative contribution to dextromethorphan metabolism by cytochrome P450 isoforms *in vitro*: can dextromethorphan be used as a dual probe for both CYP2D6 and CYP3A activities? *Drug Metabolism and Disposition* 29:1514-1520.