CHARACTERIZATION OF CYTOCHROME P450 2C8 ACTIVITY *IN VIVO*: PHARMACOGENETIC AND PHARMACOKINETIC STUDIES OF ROSIGLITAZONE METABOLISM

by

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Pharm.D., University of Pittsburgh, 2000

Submitted to the Graduate Faculty of

School of Pharmacy in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2004

UNIVERSITY OF PITTSBURGH

SCHOOL OF PHARMACY

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The Cytochrome P450 (CYP) superfamily of drug metabolizing enzymes is responsible for the metabolism of a variety of drugs and endogenous compounds. The CYP2C enzyme subfamily (CYP2C8, CYP2C9, and CYP2C19) mediates the metabolism of approximately twenty percent of these compounds. While CYP2C9 and CYP2C19 have been well characterized *in vivo*, relatively little is known about the *in vivo* contribution of CYP2C8. However, as the number of substrates and interest in polymorphic expression has grown, so too has the importance of CYP2C8. The *in vivo* relevance of CYP2C8 can be estimated with a drug predominately metabolized by this enzyme as a probe substrate. Thus, the overall purpose of this research was to investigate the utility of rosiglitazone as an *in vivo* probe of CYP2C8 activity. To accomplish this goal, we characterized the pharmacokinetics of rosiglitazone in the presence of the CYP2C8 inhibitor, trimethoprim, the CYP inducer, St. John's wort, and in subjects genotyped for variant *CYP2C8* alleles. Novel liquid chromatographic methods were developed for the determination of rosiglitazone and trimethoprim plasma concentrations with fluorescence and ultraviolet wavelength detection, respectively. *CYP2C8* genotyping was accomplished with a newly developed method based on Pyrosequencing technology, which facilitates high-throughput

analysis in a cost-effective manner. Trimethoprim was an effective inhibitor of rosiglitazone metabolism *in vitro* and it increased rosiglitazone concentrations *in vivo* by 31%. In addition, there was a strong relationship (r^2=0.97, p=0.0021) between trimethoprim plasma concentration and fold inhibition in subjects who did not carry the *CYP2C8*3* allele, suggesting genotype influences the extent of CYP2C8 inhibition. Administration of St. John's wort increased rosiglitazone clearance by 35%, but *CYP2C8* genotype did not affect the magnitude of induction. Finally, genotype did not affect basal rosiglitazone metabolism. Since changes have been observed with other CYP2C8 metabolized drugs, polymorphic effects of CYP2C8 may be substrate dependent. In conclusion, these results support the use of rosiglitazone as an *in vivo* probe of CYP2C8 activity, as it is affected by CYP2C8 inhibitors and inducers. The clinical benefits of CYP2C8 substrates may be influenced by these and other CYP2C8 modulators and therefore rosiglitazone could serve as a probe to detect these interactions.

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PREFACE

When I began this journey over four years ago, I never could have imagined where it would take me. I am grateful for everything that has happened and everyone who has helped along the way, both professionally and personally. Without these people I would not be the person I am today and would not have accomplished the following body of work.

First and foremost, I would like to thank Dr. Reggie Frye, a great role model, motivator, and advisor, who has enhanced my problem solving skills and helped me become an independent investigator. I am also grateful that he provided me the opportunity to complete my work at the University of Florida. Next I would like to thank Dr. Sam Poloyac, who has been a wonderful mentor and always reminded me to "think outside of the box." In addition I would like to thank my other committee members, Dr. Robert Ferrell, Dr. Wen Xie, and Dr. Michael Zemaitis, who have provided me with the guidance essential for scientific development.

Personally, I would like to thank God for giving me the perseverance and strength necessary to achieve this goal. I am also grateful to my parents, Mom, Dad, and Cathy, and my brothers, Larry and Dennis, for their continuous love and support. Additionally, I would like to thank the Repasky family, Dan, Jeanne, and Brooke, who have offered me not only a place in their family, but a home away from home. Finally, I would like to thank my wife, Kristy, for her unending patience and willingness to modify her career when we moved to Florida. Words cannot express my gratefulness for her daily support, inspiration, and selflessness. For this, I thank you.

Chapter 1

Introduction and Background

Cytochrome P450 Superfamily

The Cytochrome P450 (CYP) superfamily of enzymes is responsible for the breakdown of numerous xenobiotics as well as endogenous compounds (Bertz and Granneman, 1997; Rendic, 2002), including an estimated 60% to 80% of medications prescribed in the United States (Fisher *et al.*, 2001; Venkatakrishnan *et al.*, 2001). CYP enzymes catalyze Phase I biotransformation reactions, which include oxidation (e.g., hydroxylation), reduction (e.g., nitroreduction), and hydrolysis (e.g., ester hydrolysis) (Rendic, 2002). CYP enzymes are hemoproteins primarily found in the smooth endoplasmic reticulum of hepatocytes with extrahepatic expression in the intestine, lungs, kidneys, and brain (Venkatakrishnan *et al.*, 2001). These enzymes are stratified into families, subfamilies, and individual isoforms using a nomenclature system originally proposed by Nebert *et al.*, (1987), in which the name begins with the root "CYP," followed by an Arabic number indicating the family (>40% amino acid sequence homology), a letter designating the subfamily (>55% sequence homology), and an Arabic number indicating the individual gene, e.g., CYP2C8 (Nelson *et al.*, 1996).

To date there are more than 270 different *CYP* gene families, 18 recorded in mammals, which encode for 57 *CYP* genes in humans (Nebert and Russell, 2002). Although there are a large number of *CYP* genes, the *CYP1*, *CYP2*, and *CYP3* families are the primary groups involved in drug metabolism, facilitating the breakdown of drugs and endogenous compounds such as arachidonic acid and eicosanoids (Nebert and Russell, 2002). The *CYP1* family preferentially metabolizes polycyclic aromatic hydrocarbons, arylamines, and N-heterocyclics (Nebert and Russell, 2002). The *CYP2* family is the largest of the mammalian *CYP* families as it consists of 13 subfamilies and metabolizes nearly half of all frequently prescribed drugs (Nebert and Russell, 2002). Finally, the *CYP3* family consists of four enzymes, of which CYP3A4 and

CYP3A5 are the most abundantly expressed CYP enzymes in the liver (Nebert and Russell, 2002) and metabolize approximately 50% of all of the drugs broken down by CYP enzymes (Bertz and Granneman, 1997).

Genetic polymorphisms, which occur at a frequency of 1% or greater in the population (Nebert, 1999), are of increasing importance in drug metabolism and have been found in every major *CYP* gene. *CYP* variants are named according to the guidelines published by Shows *et al.*, (1987), which recommended that gene and allele (designated by Arabic numerals), are separated by an asterisk, e.g. *CYP2C9*2* (Shows *et al.*, 1987). If allelic variants share key mutations but differ in respect to other base changes, they are given the same Arabic number (allele group) followed by a capitalized letter (allele subgroup), e.g. *CYP2C19*2A* (Daly *et al.*, 1996). The first consensus or reference sequence is designated as **1*, and subsequent variants (i.e., **2*, **3*,…) are sequentially named as they are approved by the Human Cytochrome P450 Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/) (Ingelman-Sundberg *et al.*, 2000; Nebert, 2000). Polymorphisms occur primarily from single nucleotide polymorphisms (SNPs), which may be synonymous, present with no change in amino acid sequence, or nonsynonymous, exhibit variation of the amino acid sequence through substitution, addition, or deletion. Nonsynonymous SNPs may cause the creation of an alternative splice site, a frameshift mutation, gene deletion, or premature stop codon, causing non-functional alleles, e.g., *CYP2C19*3*. Alternatively, these SNPs may alter the amino acid sequence producing an allele with decreased metabolic activity, e.g., *CYP2C9*3*.

Interest in CYP activity continues to intensify due to the large number of xenobiotics and endogenous compounds metabolized by these enzymes and the influence of genetic polymorphism. Some of these enzymes, such as CYP3A, are responsible for the breakdown of

many compounds and are well studied; whereas the *in vivo* activity of other enzymes, such as CYP2C8, are relatively unknown, but are of growing importance as new substrates continue to be discovered. The purpose of the following body of work is to better characterize the role of CYP2C8 *in vivo* and to learn how modulators of CYP2C8 activity influence drug metabolism by this enzyme.

CYP2C Subfamily

The CYP2C subfamily is part of the largest family of CYP enzymes, CYP2 (Nebert and Russell, 2002), and constitutes approximately 20% of all CYP enzymes expressed in the liver (Shimada *et al.*, 1994). CYP2C enzymes contribute to the metabolism of approximately 20% of all drugs metabolized by the CYP superfamily of enzymes (Bertz and Granneman, 1997). Members of the CYP2C subfamily include CYP2C8, CYP2C9, CYP2C18, and CYP2C19. A listing of important inhibitors, inducers, and substrates for each enzyme in this subfamily is shown in Table 1. The list includes some drugs that are known to be selectively metabolized by these enzymes, such as rosiglitazone (CYP2C8) (Baldwin *et al.*, 1999), *S*-warfarin (CYP2C9) (Rettie *et al.*, 1994), and *S*-mephenytoin (CYP2C19) (Goldstein *et al.*, 1994). To date, no substrates have been found for CYP2C18.

Table 1 Substrates, inhibitors, and inducers of CYP2C8, CYP2C9 and CYP2C19

CYP2C8[(a)]	CYP2C9[(b)]	CYP2C19[(c)]
Substrates		
rosiglitazone	*S*-warfarin	*S*-mephenytoin
paclitaxel	tolbutamide	omeprazole
repaglinide	phenytoin	propranolol
amodiaquine	losartan	imipramine
chloroquine	diclofenac	proguanil activation to cycloguanil
morphine N-demethylation	piroxicam	
R-(-)-ibuprofen	*S*-(+)-ibuprofen	
Amiodarone	*R*-(-)-ibuprofen	
	glipizide	
	irbesartan	
Inhibitors[(d)]		
trimethoprim	amiodarone	fluoxetine
gemfibrozil	fluconazole	fluvoxamine
quercetin	fluvoxamine	ticlopidine
ketoconazole	sulfamethoxazole	modafinil
	modafinil	
Inducers[(d)]		
rifampin	rifampin	rifampin
hyperforin	hyperforin	hyperforin
phenobarbital	phenobarbital	phenobarbital

(a) From references: Rahman *et al.*, 1994, Hamman *et al.*, 1997, Baldwin *et al.*, 1999, Ohyama *et al.*, 2000, Li *et al.*, 2002, Bidstrup *et al.*, 2003, Projean *et al.*, 2003a, and Projean *et al.*, 2003b.

(b) From references: Goldstein and de Morais, 1994, Rettie *et al.*, 1994, Sullivan-Klose *et al.*, 1996, Hamman *et al.*, 1997, Bourrie *et al.*, 1999, Kidd *et al.*, 1999, Kidd *et al.*, 2001, Yasar *et al.*, 2001.

(c) From references: Ward *et al.*, 1989, Ward *et al.*, 1991, Goldstein *et al.*, 1994, Chang *et al.*, 1995, and Koyama *et al.*, 1997.

(d) Adapted from http://medicine.iupui.edu/flockhart/table.htm

CYP2C gene structure and regulation

The *CYP2C* subfamily is encoded on chromosome 10q24 in a cluster of 500 kb oriented in the Cen-*CYP2C18*-*CYP2C19*-*CYP2C9*-*CYP2C8*-Tel direction (Figure 1) (Gray *et al.*, 1995). The *CYP2C8* gene, which is comprised of nine exons (Figure 2), is the smallest of the *CYP2C* subfamily, spanning approximately 31 kb (Klose *et al.*, 1999). In comparison, the *CYP2C9* and *CYP2C18* genes each span more than 50 kb, and *CYP2C19* more than 90 kb (de Morais *et al.*, 1993; Klose *et al.*, 1999). The nucleotide and amino acid sequences of the *CYP2C* subfamily are highly conserved as members share greater than 80% homology (Table 2) (Goldstein and de Morais, 1994).

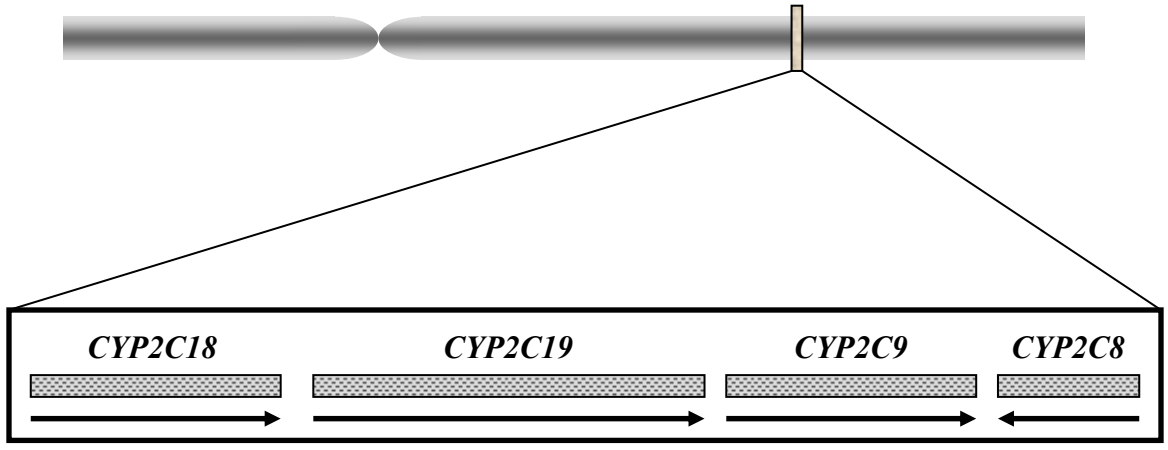


Figure 1 Schematic representation of the *CYP2C* subfamily locus on chromosome 10q24. Adapted from Gray *et al.*, 1995 and NCBI (http://www.ncbi.nlm.nih.gov/). Arrows indicate direction of transcription.

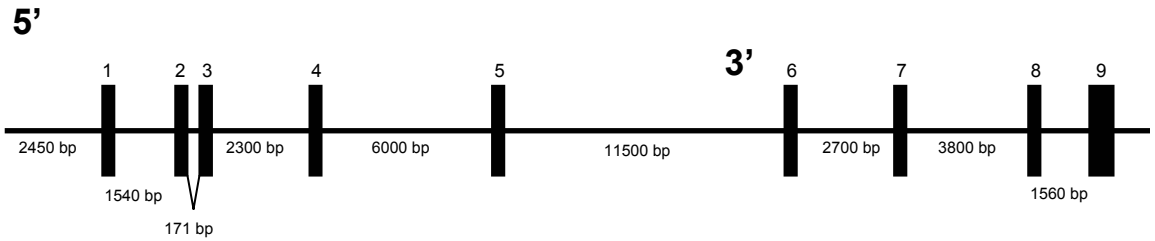


Figure 2 Schematic representation of the *CYP2C8* locus. Exons are indicated by number above block and lengths of intronic regions are shown by numbers below the figure. Adapted from Dai *et al.*, 2001.

Table 2 Nucleotide and amino acid percent homology of CYP2C cDNA. The upper value represents the nucleotide homology and the lower represents amino acid homology. Adapted from Goldstein and de Morais, 1994.

	CYP2C8	CYP2C9	CYP2C18	CYP2C19
CYP2C8	---	82.8	83.7	82.6
	---	89.4	88.7	90.6
CYP2C9	82.8	---	85.6	93.9
	89.4	---	92.7	95.7
CYP2C18	83.7	85.6	---	85.7
	88.7	92.7	---	92.7
CYP2C19	82.6	93.9	85.7	---
	90.6	95.7	92.7	---

Similar to nucleotide and amino acid sequence conservation, the regulatory and upstream regions of the *CYP2C* subfamily have significant homology. The regulatory region is well conserved between *CYP2C8* and *CYP2C9* as the 5'-flanking regions share 75% homology, but these sequences share only ~44% homology with the *CYP2C18* regulatory region (de Morais *et al.*, 1993; Goldstein and de Morais, 1994). One consensus sequence found in the upstream regions of the *CYP2C8*, *CYP2C9*, and *CYP2C18* genes is the HepG2-specific factor 1 (HPF-1) (Venepally *et al.*, 1992), which serves as a binding region for hepatocyte nuclear factor 4 (HNF-4) (Sladek *et al.*, 1990; Chen *et al.*, 1994). HPF-1 has been implicated in the hepatic regulation of *CYP2C* genes in rabbit (Chen *et al.*, 1994) and is the principal cis-acting element in positive regulation of the *CYP2C9* promoter (Ibeanu and Goldstein, 1995). Binding sites for hepatic nuclear factor 3γ (HNF-3γ) have also been found in this 5'-flanking region of *CYP2C* genes (Bort *et al.*, 2004). Other liver-enriched transcription factors that regulate CYP2C transcription such as the CCAAT/enhancer binding protein (C/EBP), hepatocyte nuclear factor 1 (HNF-1), and activating protein 1 (AP-1), have been found in the promoter regions of *CYP2C8*, *CYP2C9*, and *CYP2C18* (de Morais *et al.*, 1993; Klose *et al.*, 1999).

Analysis of the promoter region of the *CYP2C* genome has revealed binding sites for the orphan nuclear receptors, a class of nuclear receptors involved in the regulation of drug metabolizing enzymes. Nuclear receptors are ligand-activated transcription factors that are loosely categorized into two groups based on ligand status, classical and orphan nuclear receptors (Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995; Giguere, 1999). Receptors of known endocrine ligands, such as the hormones Vitamin D and all-trans-retinoic acid were identified as classical members, while receptors cloned, which had no known ligands were referred to as orphan nuclear receptors (Giguere, 1999). Two of the most notable orphan nuclear

receptors involved in the regulation of drug metabolism are the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) (Waxman, 1999; Fuhr, 2000; Moore *et al.*, 2002). Ligands of the orphan nuclear receptors, such as rifampin (a) bind to the nuclear receptor (PXR) causing (b) a translocation of the ligand-receptor complex into the nucleus, (c) heterodimerization of this ligand-receptor to the retinoid X receptor-α (RXRα), where the heterodimer (d) binds to the DNA binding region, leading to (e) increased expression of the target protein (Figure 3) (Giguere, 1999).

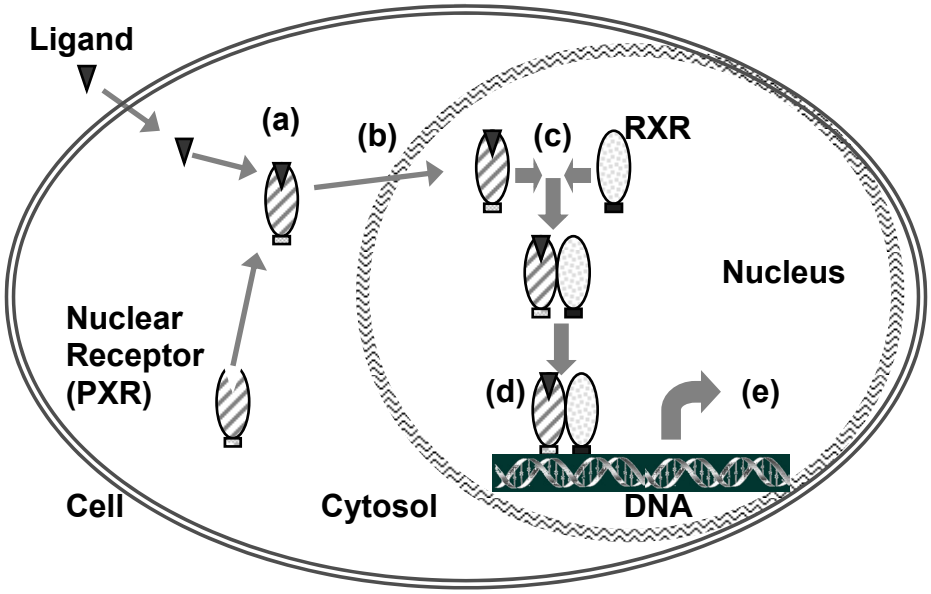


Figure 3 Mechanism of CYP activation in via the orphan nuclear receptors. (a) An exogenous ligand, such as rifampin, binds to PXR, which causes a (b) translocation into the nucleus and (c) heterodimerization with RXRα, followed by (d) the binding of the heterodimeric complex to the PXR response element (PXR-RE) in the promoter region of the DNA resulting in (e) an increase in transcription of the target protein(s).

Binding regions for the orphan nuclear receptors, known as response elements (RE) for PXR, CAR, and the glucocorticoid receptor (GR), PXR-RE, CAR-RE and GRE, respectively, have been found in the upstream regions for *CYP2C8* (Ged and Beaune, 1991), *CYP2C9* (de Morais *et al.*, 1993; Ferguson *et al.*, 2002; Chen *et al.*, 2004), and *CYP2C19* (Chen *et al.*, 2003). The GR ligand dexamethasone is a weak inducer of the CYP2C subfamily having modest effects on CYP2C8 and CYP2C19, and little effect on CYP2C9 activity (Gerbal-Chaloin *et al.*, 2001; Raucy *et al.*, 2002). Known ligands for PXR (e.g., rifampin) and CAR (e.g., phenobarbital) increase CYP2C8, CYP2C9, and CYP2C19 mRNA and protein expression in hepatocyte cultures (Gerbal-Chaloin *et al.*, 2001; Raucy *et al.*, 2002; Chen *et al.*, 2003). Further, the induction of CYP2C8 by rifampin is similar to patterns observed with CYP3A induction (Raucy *et al.*, 2002). Hyperforin, a major constituent of the herbal supplement St. John's wort, is another PXR ligand that induces CYP enzymes, including CYP2C8 (Moore *et al.*, 2000; Wentworth *et al.*, 2000; Chen *et al.*, 2004; Komoroski *et al.*, 2004). *In vivo*, rifampin induces CYP2C19, CYP2C9, and CYP2C8 activity and significantly decreases exposure of the substrates mephenytoin (Feng *et al.*, 1998), glipizide (Niemi *et al.*, 2001a), and rosiglitazone (Park *et al.*, 2004a), respectively. Further, St. John's wort increases clearance of the CYP2C9 substrate warfarin (Jiang *et al.*, 2004), and the CYP2C19 substrate mephenytoin (Wang *et al.*, 2004b).

Human CYP2C Expression and Distribution

Expression of the CYP2C subfamily is age dependent as protein concentrations are not found in fetal tissue, but rise quickly after birth, reaching approximately 50% of adult expression after one year. During this stage of development, CYP2C9 is the major isoform detected (Treluyer *et al.*, 1997). The CYP2C subfamily is primarily distributed in the liver and small intestine, but CYP2C8 and CYP2C9 exhibit a wide array of extrahepatic distribution (Table 3).

Table 3 Human distribution of CYP2C subfamily member mRNA and protein. Observations determined by Zeldin *et al.*, 1995, Hukkanen *et al.*, 1997, Mace *et al.*, 1998, McFayden *et al.*, 1998, Klose *et al.*, 1999, Glaeser *et al.*, 2002, Lapple *et al.*, 2003, and Enayetallah *et al.*, 2004.

	CYP2C8	CYP2C9	CYP2C18	CYP2C19
mRNA				
Liver	+++	++++	++	+
Small Intestine	+	+++	+++	++
Kidney	++	+	+	-
Brain	++	-	+	-
Ovaries	+	+	-	-
Lung*				
BAM	+	+	++	+
BM/PL	++	+	++	-
Adrenal gland	+	+	-	-
Mammary gland	+	-	+	-
Uterus	+	+	-	-
Testes	-	+	-	-
Prostate	-	+	-	-
Protein				
Liver	+++	++++	-	++
Small Intestine	+	+++	-	++
Kidney	++	+++	NE	NE
Adrenal gland	+	++	NE	NE
Uterus	-	+	NE	NE
Prostate	-	+	NE	NE
Salivary glands	+	++	NE	NE
Stomach	-	++	NE	NE
Spleen	-	++	NE	NE
Large intestine	++	++	NE	NE
Epidermis	-	+	NE	NE
Lymph nodes	-	+	NE	NE
Endometrium	-	+	NE	NE
Tonsils	+	+	NE	NE

Key: +: Relative abundance versus CYP2C enzymes; -: not detected in tissue; NE: not examined

*** BAM: Bronchoalveolar macrophages; BM: Bronchial Mucosa; PL: Peripheral Lung Tissue**

Hepatic mRNA expression of the CYP2C subfamily presents in the rank order of CYP2C9 > CYP2C8 > CYP2C18 > CYP2C19. Hepatic protein expression is similar, but CYP2C18 is not detectable (Lapple *et al.*, 2003). CYP2C9 and CYP2C18 mRNA are equally expressed in the small intestine (distal duodenum and proximal jejunum), followed by CYP2C19 and CYP2C8. As for intestinal protein expression, CYP2C9 is the most abundant, followed by CYP2C19, CYP2C8 (found below the limits of quantification), and CYP2C18 (not detectable) (Klose *et al.*, 1999; Glaeser *et al.*, 2002; Lapple *et al.*, 2003). Relative expression of the CYP2C enzymes is tissue specific as CYP2C9 protein is 10 fold greater in the liver compared to the kidney, and CYP2C8 mRNA is approximately 25 fold greater in the liver than the intestine (Lapple *et al.*, 2003). Further, CYP2C8 and CYP2C9 have significantly greater activity in the liver than the intestine and their activities correlate with protein expression (Lapple *et al.*, 2003).

Extrahepatic expression of the CYP2C subfamily occurs in the bronchoalveolar macrophages and lymphocytes, and peripheral lung tissue (Hukkanen *et al.*, 1997; Mace *et al.*, 1998). In the bronchial mucosa, CYP2C8 and CYP2C18 are the primary mRNA expressed followed by CYP2C9 (low) and CYP2C19 (none) (Mace *et al.*, 1998). CYP2C8 and CYP2C9 mRNA have been detected in the kidney, with CYP2C8 being the predominant isoform (Zeldin *et al.*, 1995; Klose *et al.*, 1999). Regional distribution of CYP2C8 and CYP2C9 in the kidney occurs in the proximal and distal tubules and collecting ducts, but not in the glomeruli (Enayetallah *et al.*, 2004). In the brain, CYP2C8 mRNA is detected in the cerebellum, midbrain, basal ganglia, and frontal and temporal cortices (McFayden *et al.*, 1998). CYP2C8 and CYP2C9 mRNA expression in other tissues of interest includes mammary gland, testes, adrenal gland, prostate, uterus, and ovaries (Klose *et al.*, 1999). Finally, immunohistochemical staining reveals CYP2C8 protein in salivary glands, large intestine, and tonsils and CYP2C9 protein in the

salivary glands, stomach, pancreas, tonsils, spleen, lymph nodes, epidermis, and endometrium (Enayetallah *et al.*, 2004). Overall, the CYP2C subfamily is primarily distributed in the liver but is detected in numerous tissues including the small intestine, kidney, and brain.

Polymorphic Expression of the CYP2C subfamily

Within the CYP2C subfamily considerable polymorphic expression exists, which influences the metabolism of a number of compounds. All enzymes of the CYP2C subfamily have demonstrated polymorphic expression with the primary focus on CYP2C9 and CYP2C19.

CYP2C19

The anticonvulsant mephenytoin was one of the first drugs noted to exhibit variable metabolism, which lead to the investigation of polymorphic expression of *CYP2C19*. CYP2C19, originally designated P-450$_{MP}$, has 19 allelic variants (Table 4) and mediates the 4'-hydroxylation of *S*-mephenytoin (Goldstein *et al.*, 1994). The bioconversion of *S*-mephenytoin serves as a phenotypic trait measure of CYP2C19 and is used to distinguish, extensive metabolizers (EMs), and carriers of variant alleles, poor metabolizers (PMs) (Desta *et al.*, 2002). PMs are commonly found in the Asian population with a frequency of 12 to 23% (Bertilsson *et al.*, 1992; Desta *et al.*, 2002); however, the frequency of PMs in the Caucasian population is approximately 1 to 6% (Goldstein *et al.*, 1997; Xie *et al.*, 1999; Desta *et al.*, 2002). The PM phenotype is associated with decreased metabolism of *S*-mephenytoin (de Morais *et al.*, 1994b), omeprazole (Chang *et al.*, 1995), and diazepam (Wan *et al.*, 1996). Further the PM phenotype has been implicated in pharmacodynamic response as cure rates of *Helicobacter pylori* are significantly better in PMs than EMs (Furuta *et al.*, 1998; Tanigawara *et al.*, 1999).

Table 4 *CYP2C19* allele nomenclature. Adapted from Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/)

CYP2C19 Genotype	Exon	Change		Enzyme effect	
		Nucleotide	Amino Acid	*In vitro*	*In vivo*
**1A*	--	None	None	Normal	Normal
**1B*	7	99C>T; 991A>G	$Ile^{331}Val$	Normal	Normal
**1C*	7	991A>G	$Ile^{331}Val$	Normal	Normal
**2A*	5	**1B* and 681G>A; 990C>T	splicing defect	Inactive	Inactive
**2B*	5	**1B* and 276G>C; 681G>A; 990C>T	splicing defect; $Glu^{92}Asp$	Inactive	Inactive
**3*	4	**1C* and 636G>A; 1251A>C	Stop Codon	Inactive	Inactive
**4*	1	**1B* and 1A>G	GTG Initiation Codon	ND	Inactive
**5A*	9	1297C>T	$Arg^{433}Trp$	Inactive	Inactive[(a)]
**5B*	9	**1B* and 1297C>T	$Arg^{433}Trp$	Inactive	Inactive[(a)]
**6*	3	**1B* and 395G>A	$Arg^{132}Gln$	Inactive	Inactive[(a)]
**7*	-	IVS5+2T>A	Splicing defect	ND	Inactive[(a)]
**8*	3	358T>C	$Trp^{120}Arg$	Decreased	Inactive[(a)]
**9*	3	**1B* and 431G>A	$Arg^{144}His$	Decreased	TBD
**10*	5	**1B* and 680C>T;	$Pro^{227}Leu$	Decreased	TBD
**11*	3	**1B* and 449G>A	$Arg^{150}His$	Normal	TBD
**12*	9	**1B* and 1473A>C	$Stop^{491}Cys$ (26 extra amino acids)	Unstable	TBD
**13*	8	**1C* and 1228C>T	$Arg^{410}Cys$	Normal	TBD
**14*	1	**1B* and 50T>C	$Leu^{17}Pro$	ND	TBD
**15*	1	**1C* and 55A>C	$Ile^{19}Leu$	ND	TBD
**16*	9	1324C>T	$Arg^{442}Cys$	TBD	TBD

Key: Decreased: Decreased activity versus *CYP2C19*1/*1*; **Increased:** Increased activity versus *CYP2C19*1/*1*; **TBD:** To be determined; **Inactive:** Inactive enzyme is produced; **ND:** Not determined; (a) Observed in n = 1.

The PM phenotype is primarily due to two alleles, *CYP2C19*2* and *CYP2C19*3*, which account for greater than 99% of PMs in the Asian population and approximately 88% of PMs in the Caucasian population (de Morais *et al.*, 1994a; de Morais *et al.*, 1994b; Goldstein *et al.*, 1997). *CYP2C19*2*, originally designated *CYP2C19m1* (de Morais *et al.*, 1994b), is present in frequencies of 0.25, 0.13, 0.32, and 0.23 in the African-American, Caucasian, Chinese, and Japanese populations, respectively (Goldstein *et al.*, 1997). Direct sequencing of *CYP2C19* led to the discovery of a mutation at 276G>C in 15% of individuals carrying the *CYP2C19*2* allele and was designated *CYP2C19*2B*, while the original allele was named *CYP2C19*2A* (Ibeanu *et al.*, 1998b). *CYP2C19*3*, which accounts for approximately 25% of PMs, is primarily found in the Asian population (de Morais *et al.*, 1994a). The mutation occurs from the 636G>A transversion and produces a premature stop codon and truncated CYP2C19 protein (de Morais *et al.*, 1994a). The *CYP2C19*4* allele is due to a deficient GTG initiation codon (1A>G), which produces an inactive enzyme, and has an allele frequency of 0.006 in the Caucasian population, (Ferguson *et al.*, 1998). *CYP2C19*5A* and *CYP2C19*5B* were each found in a single subject and causes reduced enzyme activity due to the 1297C>T mutation in the heme binding domain of CYP2C19. The combined allelic frequencies of *CYP2C19*5A* and *CYP2C19*5B* are 0.0025 and < 0.009 in the Chinese (Bai ethnic group) and Caucasian populations, respectively (Ibeanu *et al.*, 1998a). *CYP2C19*6* and *CYP2C19*7* have deficient catalytic activities *in vitro*, and were found in approximately 1.4% of previously studied PMs; however these alleles are extremely rare as they were not detected in a large sample of French Caucasians (n = 344) (Ibeanu *et al.*, 1998b; Ibeanu *et al.*, 1999). *CYP2C19*8* demonstrates 11 fold lower activity *in vitro* versus *CYP2C19*1B* and was demonstrated to have an allelic frequency of 0.007 in French Caucasian lung cancer patients (Ibeanu *et al.*, 1999). Of the remaining alleles, only *CYP2C19*9*,

*CYP2C19*10*, and *CYP2C19*12*, which are found in the African (African American and African Pygmies) population at allele frequencies ranging from 0.01 to 0.02, appear to have decreased *in vitro* enzyme activity (Blaisdell *et al.*, 2002). Of note, *CYP2C19*12* produces an unstable enzyme as the *CYP2C19*1* stop codon is replaced with 491Cys and 26 additional amino acids (Blaisdell *et al.*, 2002).

While numerous *CYP2C19* allelic variants have been discovered, the variants *CYP2C19*2* and *CYP2C19*3* account for the majority of PM phenotypes in the population. Further, the alleles *CYP2C19*1* through *CYP2C19*8* account for approximately 99.7% of the PM phenotype, allowing genotyping procedures to be specific (Wedlund, 2000). Other alleles, such as *CYP2C19*10* and *CYP2C19*12*, should be investigated *in vivo* to determine their contribution to the PM phenotype.

CYP2C9

Thirteen allelic variants of *CYP2C9* have been discovered to date (Table 5), with *CYP2C9*2* and *CYP2C9*3* being the primary focus of investigations. *CYP2C9*2* has an allele frequency of approximately 0.13 in the Caucasian population (Stubbins *et al.*, 1996; Dickmann *et al.*, 2001), varies from 0.01 to 0.025 in the African American population, and is rarely expressed in the Asian population (Sullivan-Klose *et al.*, 1996; Dickmann *et al.*, 2001). Similarly, *CYP2C9*3* has an allelic frequency of approximately 0.08 in the Caucasian population and 0.0125 in the African American population (Sullivan-Klose *et al.*, 1996; Dickmann *et al.*, 2001). Both of these alleles decrease CYP2C9 activity *in vitro* (Rettie *et al.*, 1994; Crespi and Miller, 1997; Yamazaki *et al.*, 1998; Takanashi *et al.*, 2000), but the *CYP2C9*3* allele has a more profound effect as it reduces the intrinsic clearance of *S*-warfarin (Sullivan-Klose *et al.*, 1996; Yamazaki *et al.*, 1998), flurbiprofen (Yamazaki *et al.*, 1998), losartan (Yasar *et al.*, 2001), and tolbutamide (Sullivan-

Klose *et al.*, 1996; Inoue *et al.*, 1997) to a much greater extent. Similar results were observed with *in vivo* studies demonstrating that the *CYP2C9*3* allele has a greater impact on the metabolism of losartan (Babaoglu *et al.*, 2004), flurbiprofen (Lee *et al.*, 2003a), and tolbutamide (Kidd *et al.*, 1999) than the *CYP2C9*2* allele. Further the *CYP2C9*2* and *CYP2C9*3* alleles affect the pharmacodynamic response of warfarin as carriers require lower daily maintenance doses and experience a greater number of bleeding complications than wild-type patients (Higashi *et al.*, 2002).

Table 5 *CYP2C9* allele nomenclature. Adapted from Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/)

***CYP2C9* Genotype**	**Exon**	**Change**		**Enzyme effect**	
		Nucleotide	**Amino Acid**	***In vitro***	***In vivo***
**1*	--	None	None	Normal	Normal
**2*	3	430C>T	$Arg^{144}Cys$	Decreased	-
**3*	7	1075A>C	$Ile^{359}Leu$	Decreased	Decreased
**4*	7	1076T>C	$Ile^{359}Thr$	Decreased	TBD
**5*	7	1080C>G	$Asp^{360}Glu$	Decreased	Decreased
**6*	5	818delA	Frameshift	ND	Inactive[(a)]
**7*	1	55C>A	$Leu^{19}Ile$	Normal	TBD
**8*	3	449G>A	$Arg^{150}His$	Increased	TBD
**9*	5	752A>G	$His^{251}Arg$	Normal	TBD
**10*	5	815A>G	$Glu^{272}Gly$	Normal	TBD
**11*	7	1003C>T	$Arg^{335}Trp$	Decreased	TBD
**12*	9	1465C>T	$Pro^{489}Ser$	Decreased	TBD
**13*	2	269T>C	$Leu^{90}Pro$	ND	Decreased[(a)]

Key: **Decreased:** Decreased activity versus *CYP2C9*1/*1*; **Increased:** Increased activity versus *CYP2C9*1/*1*; **TBD:** To be determined; **Inactive:** Inactive enzyme is produced; **ND:** Not determined; (a) Observed in n = 1.
* Promoter region mutations have been omitted to simplify the table

The *CYP2C9*4* allele has reduced CYP2C9 activity *in vitro* and is present in the Japanese population at an allele frequency of 0.02 (Ieiri *et al.*, 2000; Imai *et al.*, 2000). The *CYP2C9*5* allele is present in 0.017 of the African American population, has lower *in vitro* activity (Dickmann *et al.*, 2001), and carriers of this allele appear to demonstrate lower losartan clearance versus wild type subjects (Allabi *et al.*, 2004). A null allele, *CYP2C9*6* is detectable in the African American population at an allelic frequency of 0.006, and like *CYP2C9*5*, is not observed in Caucasian or Asian populations (Kidd *et al.*, 2001). This allele appears to cause a decrease in phenytoin clearance with associated toxicity (Kidd *et al.*, 2001) and a decrease in the clearance of losartan (Allabi *et al.*, 2004). Blaisdell *et al.*,(Blaisdell *et al.*, 2004) recently identified allelic variants designated *CYP2C9*7* through *CYP2C9*12*, which have allele frequencies ranging from 0.006 to 0.03 in African populations. Of these variants, the *CYP2C9*8*, *CYP2C9*11*, and *CYP2C9*12* alleles have altered *in vitro* metabolic activities in comparison with the wild-type allele (Blaisdell *et al.*, 2004). The final allele, *CYP2C9*13*, was identified in a Chinese population with an allele frequency of 0.01 and was associated with decreased CYP2C9 activity (Si *et al.*, 2004).

To date it appears that *CYP2C9*2* and *CYP2C9*3* are the most relevant CYP2C9 alleles in the Caucasian population. Further investigation is necessary to determine the *in vivo* relevance the other CYP2C9 variants in various populations.

CYP2C8

The importance of CYP2C8 in drug metabolism has increased over the last decade as new substrates for this enzyme have been discovered. The 6′-hydroxylation of paclitaxel has served as the classical *in vitro* marker of CYP2C8 activity (Rahman *et al.*, 1994; Dai *et al.*, 2001; Dierks *et al.*, 2001). Several CYP2C8 allelic variants that cause nonsynonymous changes in the protein have been identified (Table 6 and Figure 4).

Table 6 *CYP2C8* allele nomenclature. Adapted from Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/)

***CYP2C8* Genotype**	**Exon**	**Change**		**Enzyme effect**
		Nucleotide	**Amino Acid**	***In vitro***
**1*	--	None	None	Normal
**1B*	--	-271C>A	N/A	Normal
**1C*	--	-370T>G	N/A	Normal
**2*	5	805A>T	$Ile^{269}Phe$	Decreased
**3*	3 8	416G>A 1196A>G	$Arg^{139}Lys$ $Lys^{399}Arg$	Decreased
**4*	5	792C>G	$Ile^{264}Met$	Normal
**5*	3	475delA	Frameshift	Inactive

Key: Decreased: Decreased activity versus *CYP2C8*1/*1* ; **Increased:** Increased activity versus *CYP2C8*1/*1* ; **TBD:** To be determined; **Inactive:** Inactive enzyme is produced.

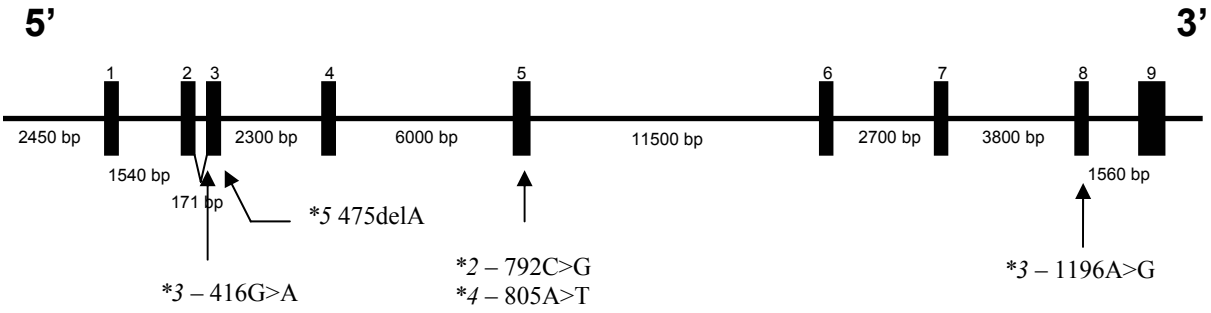


Figure 4 Schematic representation of the *CYP2C8* gene with known polymorphisms. Arrows indicate polymorphisms and respective single nucleotide polymorphisms. Adapted from Nakajima *et al.*, 2003.

The *CYP2C8*2* allele is primarily expressed in the African American population at an allele frequency of 0.18. This variation results from an 805A>T mutation and reduces the intrinsic clearance of paclitaxel (Dai *et al.*, 2001). The *CYP2C8*3* allele is primarily detected in the Caucasian population at an allele frequency ranging from 0.13 to 0.17 (Dai *et al.*, 2001; Martinez *et al.*, 2004) and is produced by two separate single nucleotide polymorphisms, 416G>A in exon 3 and 1196A>G in exon 8, which decrease paclitaxel turnover (Dai *et al.*, 2001; Soyama *et al.*, 2001). *In vivo*, the *CYP2C8*3* allele appears to increase the clearance of repaglinide (Niemi *et al.*, 2003c), but decrease the clearance of *R*-(-)-ibuprofen (Martinez *et al.*, 2004). Since the pharmacokinetic results are quite different for these compounds, further investigation of the *CYP2C8*3* allele *in vivo* is warranted. *CYP2C8*3* is also linked to the *CYP2C9*2* allele as 96% of subjects presenting with the *CYP2C8*3* variant express *CYP2C9*2* (Yasar *et al.*, 2002). The contribution of each of these variants is apparent on the metabolism of racemic ibuprofen, (Garcia-Martin *et al.*, 2004) and together may influence the metabolism of other substrates

metabolized by both CYP2C8 and CYP2C9. The *CYP2C8*4* allele, a 792C>G transversion, is found in Caucasians at an allelic frequency of approximately 0.075 (Bahadur *et al.*, 2002). The metabolism of paclitaxel (Bahadur *et al.*, 2002) *in vitro* and repaglinide *in vivo* (Niemi *et al.*, 2003c) are not significantly different than metabolism by the wild type allele, suggesting that this variant does not alter CYP2C8 activity. However, it should be noted that in the repaglinide evaluation, only three subjects genotyped as *CYP2C8*1/*4* were compared to nineteen *CYP2C8*1/*1* subjects and there was large variability in clearance. A larger and more balanced study design may be needed to determine the *in vivo* relevance of carrying the *CYP2C8*4* allele. The deletion of adenine at position 475 produces a rare frameshift mutation, *CYP2C8*5*, that has an allele frequency of approximately 0.0025 in the Japanese population (Nakajima *et al.*, 2003). This deletion is thought to cause the initiation of a stop codon at codon 177 (Soyama *et al.*, 2002b), and has been implicated in toxicity associated with the CYP2C8 substrate cerivastatin (Nakajima *et al.*, 2003; Ishikawa *et al.*, 2004). Two other variants are found upstream of the CYP2C8 translation region, *CYP2C8*1A* and *CYP2C8*1B*, which have no effect on CYP2C8 expression (Bahadur *et al.*, 2002). To date the *CYP2C8*2*, *CYP2C8*3*, and *CYP2C8*4* alleles have been investigated *in vitro*, but the *in vivo* effects are not completely understood. To investigate the significance of these mutations, a probe drug of CYP2C8 activity would be useful.

Probe Drugs

Substrates metabolized primarily by an individual enzyme are useful for estimating enzyme activity. When used for *in vitro* investigations, these substrates may be drugs, paclitaxel (CYP2C8) (Baldwin *et al.*, 1999; Masimirembwa *et al.*, 1999), endogenous compounds, testosterone (CYP3A) (Masimirembwa *et al.*, 1999), or chemical entities synthesized to be

enzyme selective, 7-ethoxy-4-trifluoromethylcoumarin (CYP2B6) (Jinno *et al.*, 2003). When used for *in vivo* investigations, synthesized chemical entities and endogenous compounds (e.g., testosterone) have many substantial drawbacks, the most important being safety. These compounds may not be approved for human use or safe to administer to specific groups, such as women or children. One approach to estimate *in vivo* CYP activity is to employ a probe drug, a substrate primarily metabolized by an individual enzyme. A probe drug ideally would possess the following characteristics: safety, a relatively short half-life, metabolism exclusively mediated by the pathway of interest (and that pathway is the major route of metabolism), and rapid excretion of the metabolite (Watkins, 1994). To evaluate hepatic CYP activity, considerations for a probe substrate include a low extraction ratio, the lack of first pass metabolism, and lack of interindividual variability of the volume of distribution and protein binding (Watkins, 1994). Several examples of probe drugs include caffeine (CYP1A2) (Sharma *et al.*, 2004), *S*-mephenytoin and omeprazole (CYP2C19) (Chang *et al.*, 1995; Frye *et al.*, 1997; Chainuvati *et al.*, 2003), tolbutamide (CYP2C9) (Lee *et al.*, 2003b), metoprolol (CYP2D6) (Sharma *et al.*, 2004), chlorzoxazone (CYP2E1) (Frye *et al.*, 1997; Poloyac *et al.*, 1999), and midazolam (CYP3A) (Lee *et al.*, 2002).

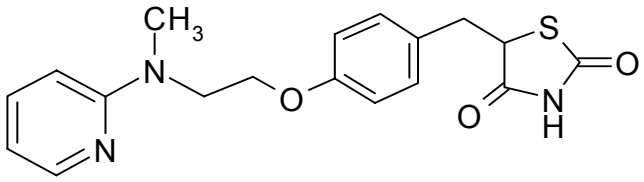
Several candidate drugs exist to evaluate *in vivo* CYP2C8 activity including rosiglitazone, repaglinide, and paclitaxel. Although paclitaxel is commonly used to assess *in vitro* activity (Dai *et al.*, 2001), it is an antineoplastic agent that can not be administered safely to healthy volunteers (Sonnichsen *et al.*, 1995). Repaglinide is a safer alternative to paclitaxel, but it is metabolized into several metabolites, which are produced by both CYP2C8 and CYP3A (Bidstrup *et al.*, 2003). Since repaglinide is metabolized by CYP3A, drug interactions related to CYP2C8 and CYP3A, such as induction by rifampin (Raucy *et al.*, 2002), would complicate

interpretation. Rosiglitazone may be suitable for use as an *in vivo* CYP2C8 probe since it has a favorable safety and pharmacokinetic profile and it is primarily metabolized by CYP2C8 (Baldwin *et al.*, 1999).

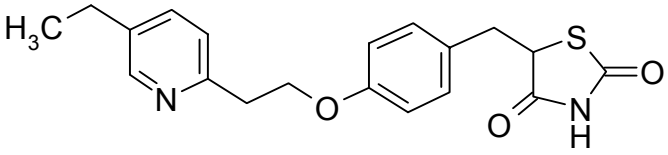
Rosiglitazone

Rosiglitazone (Avandia®) is an antihyperglycemic medication used in the treatment of type 2 diabetes mellitus, and is a member of the thiazolidinedione class of drugs, which are commonly referred to as "the glitazones" (Wagstaff and Goa, 2002). Members of this class of antihyperglycemic agents include rosiglitazone, pioglitazone, and troglitazone (Figure 5); (troglitazone was withdrawn from the market by the U.S. Food and Drug Administration (FDA) due to hepatic toxicity). Thiazolidinedione medications exert their clinical effect through the Peroxisome Proliferator-Activated Receptor-γ (PPARγ). Activation of this receptor by the thiazolidinediones leads to increased glucose utilization and metabolism, increased fatty acid utilization, decreased endogenous glucose production, and decreased tissue resistance to glucose (Mayerson *et al.*, 2002). This class of medications does not trigger the pancreas to secrete insulin like other antihyperglycemic agents, such as the sulfonylureas (e.g., glipizine or tolbutamide), and therefore does not cause hypoglycemia in healthy volunteers (Cox *et al.*, 2000; Inglis *et al.*, 2001; Miller *et al.*, 2002).

Rosiglitazone



Pioglitazone



Troglitazone

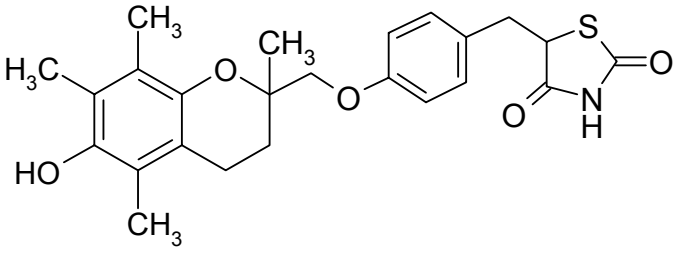


Figure 5 Structures of the thiazolidinedione class of antihyperglycemic agents.

For the treatment of type 2 diabetes mellitus, the standard dosage begins at 4 mg daily, but can be increased to 8 mg daily. Rosiglitazone can be used as monotherapy as well as adjuvant therapy with insulin or metformin (Wagstaff and Goa, 2002). The most common adverse events associated with daily rosiglitazone administration include upper respiratory tract infection, injury, and headache (Wagstaff and Goa, 2002); however, peripheral edema is a concern after repeated administration (Thomas and Lloyd, 2001; Niemeyer and Janney, 2002; Idris *et al.*, 2003). Unlike the hepatic problems that plagued troglitazone, very few cases of liver failure have been reported (Al-Salman *et al.*, 2000; Lee *et al.*, 2002) and the incidence of elevated transaminase levels is low (Wagstaff and Goa, 2002). Further, retrospective analysis of over 6000 patients and subjects that received rosiglitazone in controlled trials concluded that no major

cases of hepatic toxicity occurred (Lebovitz *et al.*, 2002). Rosiglitazone pharmacokinetic parameters are as follows: half-life of approximately 3 to 4 hours, albumin protein binding of 0.99, and bioavailability of approximately 0.99 (Wagstaff and Goa, 2002). Administration in the presence of food can prolong the time until peak plasma concentration, but there is no effect on oral bioavailability (Freed *et al.*, 1999). End stage renal disease and age do not affect rosiglitazone absorption and disposition (Thompson-Culkin *et al.*, 2002; Wagstaff and Goa, 2002). Rosiglitazone is completely metabolized, primarily, into two major metabolites, *para*-hydroxyrosiglitazone and *N*-desmethylrosiglitazone (Figure 6). The sulfate conjugate of the former, *para*-O-sulfate rosiglitazone, and *N*-desmethylrosiglitazone account for > 80% of all metabolites isolated in plasma. After single dose administration, *para*-O-sulfate rosiglitazone accounts for approximately 16% of the dose excreted in the urine and no unchanged parent drug is found (Cox *et al.*, 2000). In terms of PPARγ activation, rosiglitazone metabolites are 55% less potent than the parent compound (Wagstaff and Goa, 2002).

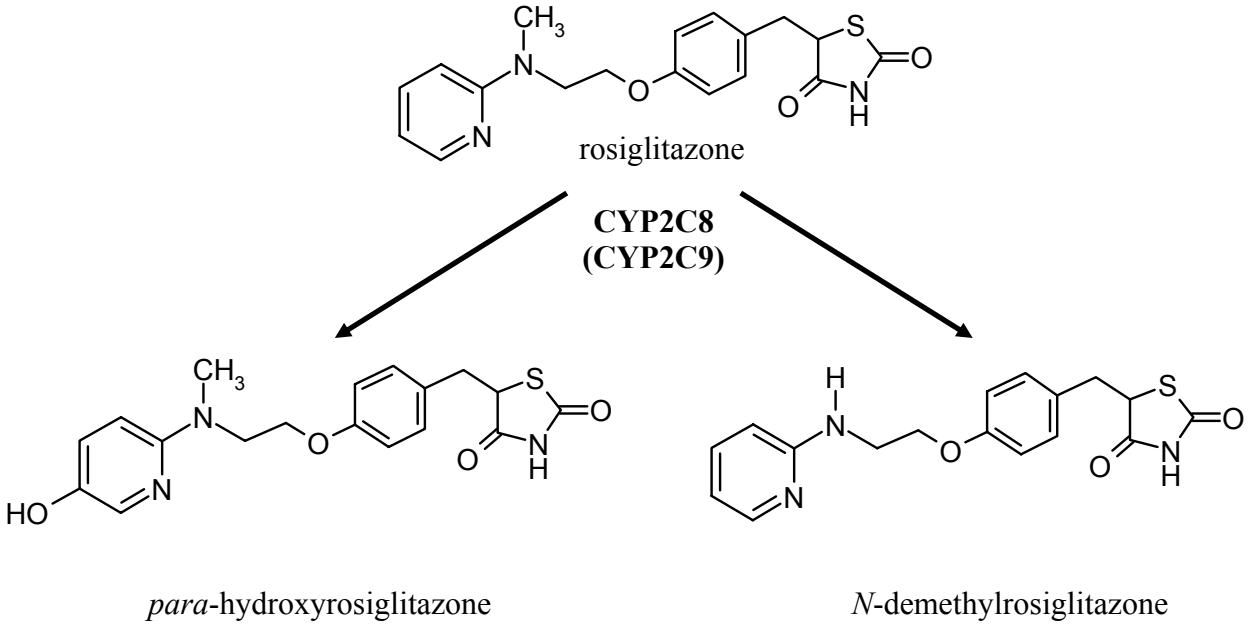


Figure 6 Metabolism of rosiglitazone into two major metabolites. Biotransformation of rosiglitazone to *para*-hydroxyrosiglitazone and *N*-desmethylrosiglitazone is performed primarily by CYP2C8, with a minor contribution of CYP2C9 (Baldwin *et al.*, 1999).

Drug interaction studies have demonstrated that rosiglitazone pharmacokinetic parameters were not influenced by acarbose (Miller *et al.*, 2001), ranitidine (Miller *et al.*, 2002), sucralfate (Rao *et al.*, 2002), and metformin (Di Cicco *et al.*, 2000a). However, the CYP inhibitors ketoconazole and gemfibrozil decrease the clearance of rosiglitazone (Niemi *et al.*, 2003a; Park *et al.*, 2004b) and the CYP inducer rifampin substantially increases rosiglitazone clearance (Park *et al.*, 2004a). Contrary to *in vitro* studies that implicate the thiazolidinediones, troglitazone, pioglitazone and rosiglitazone, as inducers of CYP3A activity (Sahi *et al.*, 2003), rosiglitazone has no clinical effect on the metabolism of CYP3A substrates, nifedipine (Harris *et al.*, 1999), ethinylestradiol, and norethindrone (Inglis *et al.*, 2001). Further, rosiglitazone does not affect exposure to

metformin (Di Cicco *et al.*, 2000a), or the P-glycoprotein substrate, digoxin (Di Cicco *et al.*, 2000b). Therefore, based on the safety and pharmacokinetic profile, rosiglitazone may serve as a good CYP2C8 probe.

Summary and Introduction to Dissertation

CYP2C is an important enzyme subfamily in drug metabolism as it is widely distributed in the body, metabolizes approximately 20% of known CYP substrates, and is influenced by a number of CYP inhibitors and inducers. While a multitude of studies have investigated CYP2C9 and CYP2C19 activity *in vivo*, little work has focused on CYP2C8. Since rosiglitazone is primarily metabolized by CYP2C8 and has a favorable pharmacokinetic and safety profile, it would be advantageous to investigate its utility as an *in vivo* probe of CYP2C8 activity.

The following research will evaluate rosiglitazone as a CYP2C8 probe through the development of assay methods instrumental in determining CYP2C8 genotype and plasma concentrations of rosiglitazone and trimethoprim, as well as clinical studies to evaluate rosiglitazone disposition in the presence of trimethoprim (inhibitor), St. John's wort (inducer), and CYP2C8 allelic variants. The aim of Chapter 2 was to develop a simplified method to determine rosiglitazone concentrations in human plasma. Development of an improved method for the determination of trimethoprim concentrations in human plasma is presented in Chapter 3. The goal of Chapter 4 was to utilize Pyrosequencing technology as a means to determine *CYP2C8* genotype in a high-throughput design. The aim of Chapter 5 was to investigate the effect of the CYP2C8 inhibitor trimethoprim on rosiglitazone metabolism *in vitro* and *in vivo*. The objective of Chapter 6 was to evaluate the effect of the herbal supplement, St. John's wort, on the *in vivo* metabolism of rosiglitazone. The goal of Chapter 7 was to characterize the pharmacokinetics of rosiglitazone in subjects carrying the variant alleles, *CYP2C8*2*,

*CYP2C8*3*, and *CYP2C8*4*. The work herein was performed to demonstrate the utility of rosiglitazone as an *in vivo* probe of CYP2C8 activity, through the evaluation of rosiglitazone metabolism in the presence of genetic and non-genetic factors that affect CYP2C8 activity.

Chapter 2

Simplified Method for Determination of Rosiglitazone in Human Plasma

[Hruska MW and Frye RF. Simplified Method for Determination of Rosiglitazone in Human Plasma. *J Chromatogr B* 2004;803(2): 317-20.]

Abstract

Rosiglitazone is a thiazolidinedione antihyperglycemic drug used in the treatment of type 2 diabetes mellitus. Rosiglitazone is extensively metabolized by cytochrome P450 2C8 and may have some utility as an *in vivo* probe for this enzyme. A liquid chromatographic method using sensitive fluorescence detection and simplified sample processing involving protein precipitation with acetonitrile was developed. The isocratic mobile phase consisted of 10 mM sodium acetate-ACN (pH 5; 60:40, v/v) and was delivered at a flow rate of 1 ml/min to an Alltima phenyl column (250 mm x 4.6 mm, 5 micron). Detection was by fluorescence at (EX/EM) 247/367 for rosiglitazone and 235/310 for the internal standard betaxolol. Intra- and inter-day precision ranged from 3.1% – 8.5% and 2.3% – 5.7%, respectively. No endogenous interference was observed with either rosiglitazone or the internal standard. The assay is simple, economical, precise, and is directly applicable to human pharmacokinetic studies involving single dose rosiglitazone administration.

Introduction

Rosiglitazone (Figure 7) is a thiazolidinedione antihyperglycemic drug used in the treatment of type 2 diabetes mellitus. Thiazolidinedione antihyperglycemic agents exert their effect through the Peroxisome Proliferator-Activated Receptorγ (PPARγ), which facilitates the expression of genes responsible for glucose and lipid metabolism (Balfour and Plosker, 1999). Rosiglitazone has a half-life in humans of approximately 3 to 4 hours, a volume of distribution of approximately 18 liters, and it is 99.8% bound to plasma proteins (Balfour and Plosker, 1999). Rosiglitazone undergoes extensive metabolism with essentially no parent drug excreted unchanged in the urine. The two major metabolites of rosiglitazone isolated via microsomal and animal studies are *para*-hydroxyrosiglitazone and *N*-desmethylrosiglitazone, both of which are produced by cytochrome P450 (CYP) 2C8 (Bolton *et al.*, 1996; Baldwin *et al.*, 1999).

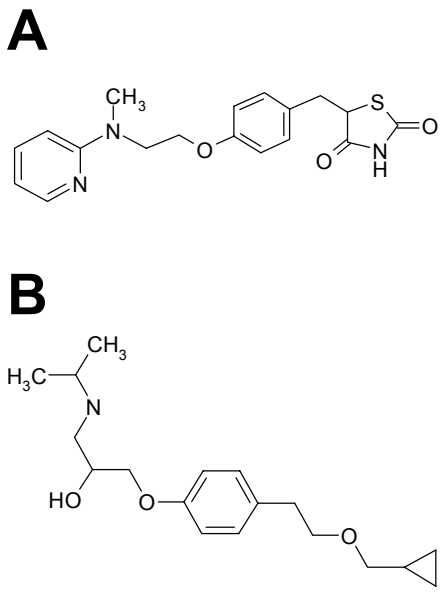


Figure 7 Structures of (A) Rosiglitazone and (B) Betaxolol (internal standard).

Since rosiglitazone is predominantly metabolized by CYP2C8, it may have potential use as an *in vivo* probe of this enzyme. Availability of a simple method for determination of rosiglitazone in human plasma would aid in the evaluation of rosiglitazone as a CYP2C8 probe. Several methods have been published but each has drawbacks limiting ease of use. Muxlow, *et al.*, developed a method using the automated sequential trace enrichment of dialysates (ASTED) system combined with fluorescence detection (Muxlow *et al.*, 2001). A drawback of this method is that it requires a specialized extraction system (ASTED) and it uses a synthesized internal standard, both of which are not readily available. Also, the linear standard curve range extended only to 100 ng/ml, which is not ideal for human pharmacokinetic studies of rosiglitazone. After a single dose of rosiglitazone, maximum plasma concentrations exceed 600 ng/ml (Cox *et al.*, 2000). Mamidi *et al.*, reported a method that is linear up to 1000 ng/ml, but uses an internal standard that is not commercially available (Mamidi *et al.*, 2002). The method of Kolte *et al.*, utilizes HPLC linked to ultraviolet detection and a commercially available compound (pioglitazone) as the internal standard (Kolte *et al.*, 2003). However, the method requires a large sample volume (1 ml) and a prolonged analysis time of 25 min per sample. Thus, the goal of this work was to establish a method that has a simple and inexpensive extraction method, requires a small plasma sample volume, and has a readily available internal standard. In this paper, we introduce an HPLC fluorescence method with simplified sample processing to quantify rosiglitazone in human plasma. The method was used to determine rosiglitazone plasma concentrations after single-dose administration.

Experimental

Chemicals and Reagents

Rosiglitazone reference standard (≥ 98%) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Betaxolol reference standard (100%) was obtained from United States Pharmacopeia (Rockville, MD, USA). Sodium Acetate and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Blank human plasma was purchased from the Central Blood Bank of Pittsburgh (Pittsburgh, PA, USA). HPLC grade deionized water was obtained from an in-house Millipore (Billerica, MA, USA) water system.

Instrumentation and Chromatographic Conditions

The HPLC system consisted of a Waters 501 Pump coupled with a Waters model 2475 fluorescence detector (Waters Corp., Milford, MA). Detection and quantification was performed using Millennium32 software version 3.1 (Waters Corp., Milford, MA). Separation was achieved with a 250 mm x 4.6 mm, 5μm Alltima phenyl column (Alltech Associates Inc., Deerfield, IL) and an isocratic mobile phase of 10 mM sodium acetate (pH 5)-ACN (60:40, v/v) delivered at a flow rate of 1 ml/min. Rosiglitazone was monitored at λex of 247, λem of 367 and betaxolol was monitored at λex of 235 and λem of 310. During each sample run, λex = 247 and λem = 367 was monitored from 0 to 7 and 10 to 15 minutes, and λex = 235 and λem = 310 was monitored from 7 to 10 minutes. The total run time was 15 min.

Standard Preparation

Rosiglitazone stock solution was prepared at a concentration of 1 mg/ml in ethanol. Dilutions that were prepared in ethanol at concentrations of 200ng/ml, and 2, 10, and 20 μg/ml were used to prepare calibration standards and quality control (QC) samples. The internal standard (IS)

betaxolol was dissolved in methanol to prepare a 25 μg/ml stock solution. Blank plasma was spiked with appropriate stock solutions and brought up to 200 μL prior to processing.

Sample Preparation

IS (10 μL or 250 ng) was added to plasma samples (200 μL) in microcentrifuge tubes and vortexed briefly. ACN (600 μL) was then added to each sample, vortexed for 2 min, and centrifuged at 3,000 g for 10 min. Supernatant was evaporated using nitrogen gas in a heating block set at 45°C. Dried samples were reconstituted with 200 μL of mobile phase and a 75 μL aliquot was injected onto the column.

Calibration and Linearity

Calibration curves consisted of seven standard concentrations of rosiglitazone in human plasma: 5, 10, 50, 100, 250, 500, and 1000 ng/ml. Duplicate calibration curves were analyzed daily for 3 days, with the lowest concentration (5 ng/ml) prepared in triplicate. For each curve, the rosiglitazone peak height to IS peak height ratio was calculated and plotted against nominal rosiglitazone concentrations. Calibration curves for rosiglitazone were constructed by weighted ($1/y^2$) linear regression analysis.

Precision and Accuracy

Precision and accuracy were determined by the analysis of rosiglitazone QC samples spiked at concentrations of 40, 400, and 750 ng/ml. Replicate QC samples (n = 12) were analyzed on Day 1 to determine intra-day precision and accuracy. Inter-day precision and accuracy were determined by replicate QC samples on Day 1 (n =12), Day 2 (n = 6), and Day 3 (n= 6), for a total of n = 24 QC samples at each concentration. Mean, standard deviation, and relative

standard deviation (RSD) were calculated from QC values and used in the estimation of intra- and inter-day precision. Accuracy (bias) is expressed as the percent difference between the calculated mean concentration relative to the nominal concentration.

Selectivity and Stability

Selectivity was assessed by processing six separate sources of blank plasma. Blank plasma samples were processed in duplicate and compared to plasma spiked with the lowest rosiglitazone standard. Sample carryover was determined by inserting vials of blank mobile phase in random positions throughout the third validation. Batches of high and low QCs were prepared and subjected to three freeze-thaw cycles (-80°C to room temperature) prior to processing and analysis. After each freeze thaw cycle, aliquots were extracted and analyzed. To determine stability of processed samples, high and low QC samples were prepared and subjected to repeated analysis over a 24 h period post-extraction.

Extraction Efficiency

Extraction efficiency was determined by comparing the response obtained from extracted QC samples versus the response observed after direct injection of reference samples. Reference samples consisted of water spiked with appropriate amounts of rosiglitazone. Responses obtained from reference samples were defined as 100%.

Application to plasma sampling

Rosiglitazone pharmacokinetics were evaluated in a healthy volunteer. The protocol was approved by the University of Pittsburgh Institutional Review Board and signed informed consent was obtained. Rosiglitazone 8mg (Avandia®, GlaxoSmithKline, Philadelphia, PA, USA) was administered orally with 8 ounces of water after an overnight fast. Plasma was

collected for 24 h, and stored at -20°C until analyzed. Plasma concentrations of rosiglitazone were determined as described above. The maximum observed rosiglitazone concentration (Cmax) and the time at which Cmax was observed (Tmax) are reported. The terminal elimination rate constant (λz) was estimated by nonlinear least squares regression analysis of the terminal portion of the concentration-time curve. The apparent elimination half-life was calculated as 0.693/λz.

Results

Chromatography

Representative chromatograms of plasma samples are depicted in Figure 8. Figure 8A depicts a blank plasma sample and a plasma sample spiked with rosiglitazone 5 ng/ml (LOQ); Figure 8B shows a plasma sample obtained 1 h after a single dose of rosiglitazone 8 mg. Retention times were approximately 7.9 min and 13.2 min for IS and rosiglitazone, respectively. The peaks of interest were well separated and free from interference with endogenous substances.

Precision, Linearity, and Accuracy

Linear calibration curves were obtained for rosiglitazone over the concentration range of 5 – 1000 ng/ml; the mean regression equation was: $Y = [0.0058 \pm 0.0020]X + [0.0022 \pm 0.0018]$. The correlation coefficients calculated for each run were > 0.99. Intra- and inter- day precision (RSD) was ≤ 8.5% and accuracy was within ± 11.1% (Table 7). The signal to noise ratios for rosiglitazone at 5 ng/ml and the I.S. at 250 ng were greater than 5:1 (Figure 8A).

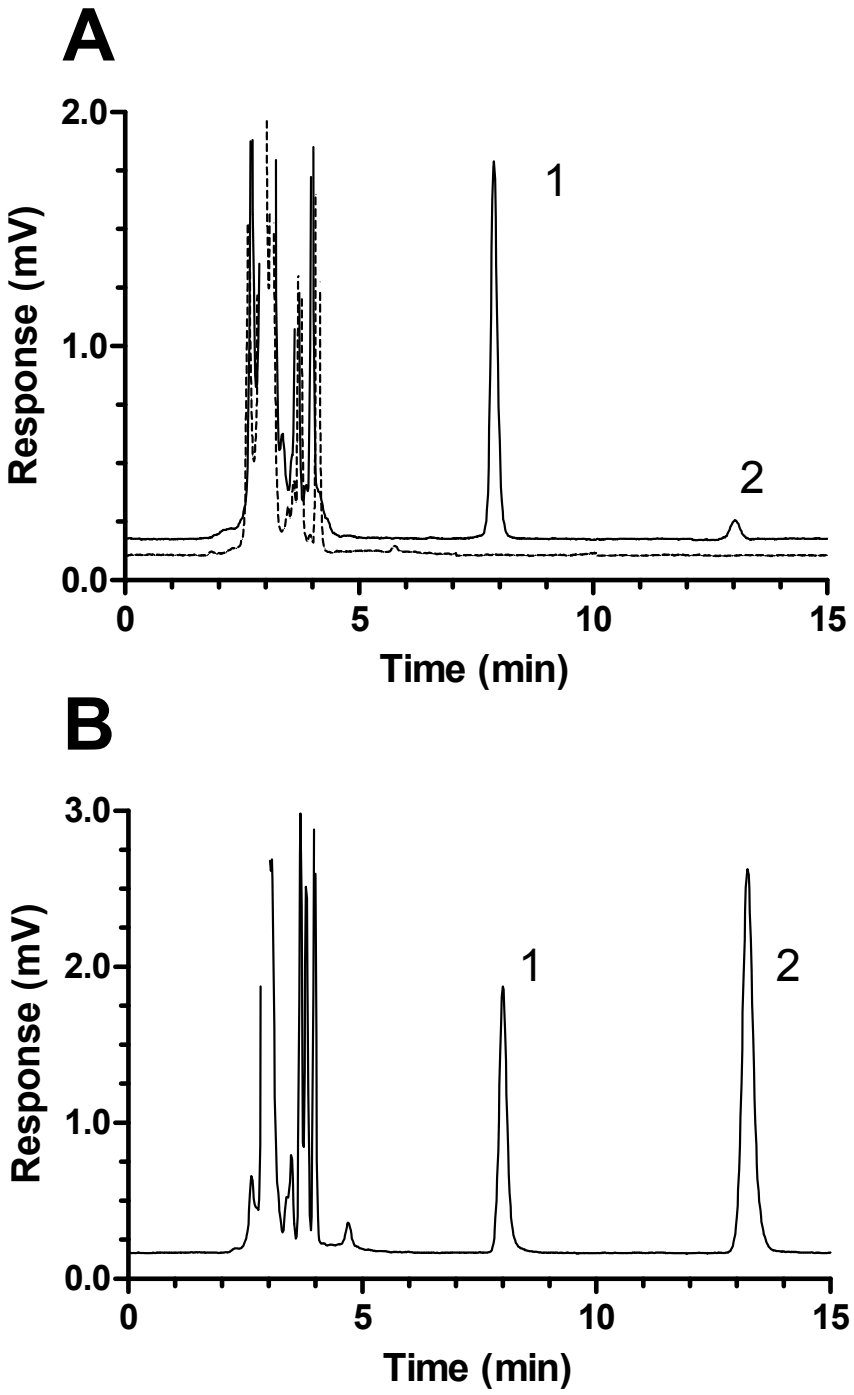


Figure 8 Representative chromatographs of (A) extracted blank plasma (----) and plasma spiked (—) at LOQ concentration of rosiglitazone 5 ng/ml (offset = 0.05mV); (B) rosiglitazone in plasma at 1 hour after single dose rosiglitazone 8 mg administration (concentration 210.2 ng/ml); Peaks (1) I.S., (2) Rosiglitazone.

Table 7 Intra- and inter-day precision and accuracy of rosiglitazone in plasma.

	Concentration (ng/ml)		% RSD	% Bias
	Spiked	**Observed (mean ± S.D.)**		
Intra-assay Precision (n = 12)				
Quality Controls				
	40	38.5 ± 1.2	3.1	-3.8
	400	367.2 ± 18.9	5.1	-8.2
	750	759.6 ± 64.6	8.5	1.3
Inter-assay Precision (n = 24)				
Quality Controls				
	40	38.1 ± 0.9	2.3	-4.8
	400	355.8 ± 16.1	4.5	-11.1
	750	706.0 ± 40.5	5.7	-5.9
Standards				
	5	5.1 ± 0.3	6.0	1.1
	10	10.0 ± 0.6	5.8	-0.4
	50	50.5 ± 2.5	4.9	1.0
	100	98.7 ± 3.9	4.0	-1.3
	250	246.4 ± 5.6	2.3	-1.5
	500	531.6 ± 43.8	8.2	6.3
	1000	982.1 ± 28.2	2.9	-1.8

Selectivity, Stability, and Recovery

No endogenous interference with rosiglitazone or IS was observed in six different sources of blank plasma. There was no evidence of sample carryover. Rosiglitazone and IS were both stable in the injection solvent at room temperature for at least 24 h prior to injection. Samples subjected to three freeze-thaw cycles showed no degradation of rosiglitazone. Extraction recovery determined at the three QC concentrations was ~100% (Table 8).

Table 8 Extraction recovery of rosiglitazone and internal standard from human plasma (n = 3).

Spiked Concentration	% Recovery	% RSD
Rosiglitazone (ng/ml)		
40	105.7 ± 5	4.68
400	113.8 ± 2	1.36
750	105.6 ± 1	0.94
Internal Standard (μg/ml)		
25	96.7 ± 6	6.40

Plasma Sampling

The log concentration-time profile for a healthy subject administered rosiglitazone 8 mg as a single dose is illustrated in Figure 9. The observed C_{max} was 697.3 ng/ml, which occurred at a t_{max} of 0.5 h; the observed $t_{1/2}$ was 3.9 h.

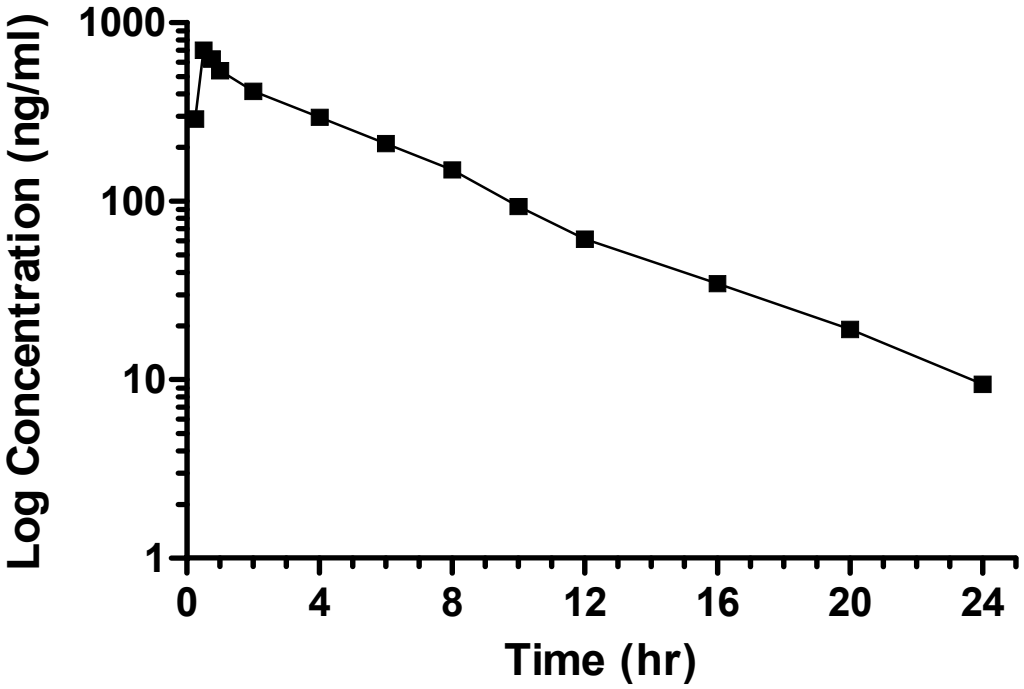


Figure 9 Log-concentration – time profile for subject administered single dose rosiglitazone (8 mg).

Discussion

The method presented here allows for simple and economical detection of rosiglitazone in small volumes of human plasma, using protein precipitation coupled with sensitive fluorescent detection. The extraction technique does not require additional expensive solid phase extraction equipment such as the ASTED system (Cox *et al.*, 2000; Muxlow *et al.*, 2001). Further, this method uses a commercially available fluorescent compound as an internal standard, rather than a synthesized thiazolidinedione-like structure as an internal standard (Cox *et al.*, 2000; Mamidi *et al.*, 2002). Although the method developed by Muxlow *et al.* (Muxlow *et al.*, 2001), was utilized in the clinical development of rosiglitazone, the standard range had a maximum concentration of 100 ng/ml. Single dose rosiglitazone yields maximum plasma concentrations well above a threshold of 100 ng/ml, as shown by the data in Figure 9 and other studies (Cox *et al.*, 2000; Mamidi *et al.*, 2002), so an additional dilution step would be required to fit within this standard curve range. A more recent method described by Kolte *et al.* (Kolte *et al.*, 2003), used a commercially available product as an internal standard but required a much larger volume of plasma due to less sensitive ultraviolet detection. In the method presented here, smaller volumes of plasma can be analyzed since we used fluorescence detection, which has greater sensitivity than ultraviolet detection. By extracting smaller plasma samples, less organic solvents may be used in extraction, yielding less cost and less time drying extracted samples. The analysis time is also much shorter in this assay, 15 min versus 25 min, which cuts down on overall time of sample analysis. Since there is no endogenous interferences in the regions where rosiglitazone and the IS elute (Figure 8), the analysis time could be decreased even further by using a shorter column.

In this paper, we have introduced a simplified HPLC fluorescence method to quantify rosiglitazone in human plasma. The method provides an economical, simple alternative to the methods previously published and is suitable for the determination of rosiglitazone plasma concentrations after single-dose administration.

Acknowledgements

This project was supported in part by NIH Research Grant R01 MH63458, funded by the National Institute of Mental Health and the Office of Dietary Supplements, and NIH/NCRR/GCRC#5M01RR00056.

Chapter 3

Determination of Trimethoprim in Low-Volume Human Plasma by Liquid Chromatography

[Hruska MW and Frye RF. Determination of Trimethoprim in Low-Volume Human Plasma by Liquid Chromatography. *J Chromatogr B* 2004;807(2): 301-5.]

Abstract

Trimethoprim is an anti-infective agent used in the treatment of urinary and respiratory tract infections and mild to moderate pneumocystis carinii pneumonia. Trimethoprim is also a selective *in vitro* inhibitor of cytochrome P450 2C8 and may have utility as an *in vivo* inhibitor of this enzyme. A simplified high performance liquid chromatography (HPLC) method was developed to determine trimethoprim in human plasma. Samples are processed by protein precipitation with perchloric acid and separation is achieved on a Synergi Polar-RP column (4 micron, 150 mm x 4.6 mm) using a mobile phase consisting of 50 mM ammonium formate-acetonitrile-methanol (pH = 3.0; 90:6:4, v/v/v). Detection is monitored at 280 nm. Intra- and inter-day precision ranged from 1.1% – 1.9% and 0.9% – 4.1%, respectively. The assay is simple, economical, precise, and is directly applicable to human studies involving steady state trimethoprim pharmacokinetics.

Introduction

Trimethoprim (Figure 10), a dihydrofolate reductase inhibitor, is an anti-infective agent used predominantly in the treatment of urinary and respiratory tract infections (Dollery, 1999). Trimethoprim is also used in combination with sulfamethoxazole or dapsone to treat mild to moderate cases of pneumocystis carinii pneumonia (PCP) (Korraa and Saadeh, 1996). Treatment of PCP requires trimethoprim doses of 15 - 23 mg/kg/day, which yields mean plasma concentrations of 6.7 to 18.4 μg/ml (23.1 – 63.4 μM) (Lee *et al.*, 1989; Klinker *et al.*, 1998). The frequency of severe adverse effects including neutropenia and thrombocytopenia increases when trimethoprim plasma concentrations exceed 8 μg/ml (Klinker *et al.*, 1998). Trimethoprim is well-absorbed after oral administration (bioavailability ~95%) and achieves peak plasma concentrations 1-4 hours after single dose administration. It has a half-life of ~10 hours, protein binding of 44%, a volume of distribution ranging from 70 -100 L, and it is extensively eliminated in the urine as unchanged drug (Dollery, 1999).

In addition to its therapeutic applications, trimethoprim is a selective *in vitro* inhibitor of cytochrome P450 (CYP) 2C8 (K_i = 32 μM; 1μM = 290.3 μg/L) (Wen *et al.*, 2002), and may be useful as an *in vivo* inhibitor of this enzyme. Peak plasma concentrations after trimethoprim 200 mg given twice daily are approximately 20 μM (Moore *et al.*, 1996). Wen *et al.* (2002), predicted based on expected plasma concentrations, that *in vivo* inhibition of CYP2C8 would be approximately 80% assuming that hepatic trimethoprim concentrations would be approximately 130 μM, which is based on the liver to plasma partitioning ratio of 6.5:1 observed in monkeys (Craig and Kunin, 1973). These observations suggest that trimethoprim may be useful as a selective enzyme inhibitor *in vivo*, but further study is required.

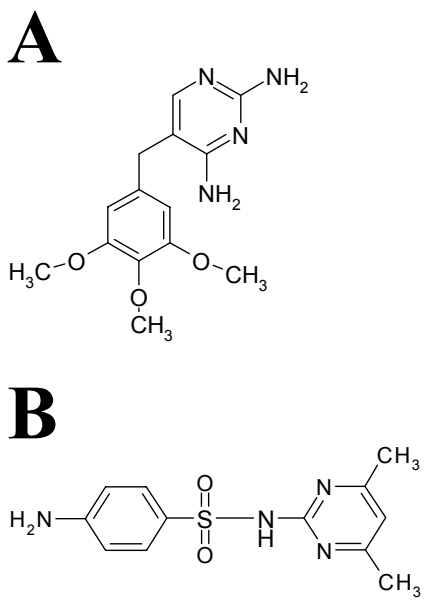


Figure 10 (A) Trimethoprim (B) Sulfamethazine, Internal Standard (IS)

There is a need to measure trimethoprim in plasma to ensure effective concentrations are achieved in patients being treated for PCP and also to aid in the evaluation of trimethoprim as an *in vivo* inhibitor of CYP2C8. Thus, a simple, efficient method for determination of trimethoprim in plasma is desired. Several methods for the determination of trimethoprim in human plasma have been reported (Gochin *et al.*, 1981; DeAngelis *et al.*, 1990; Laizure *et al.*, 1990; Avgerinos *et al.*, 1991; Pokrajac *et al.*, 1998; Ronn *et al.*, 1999). In general they require large (1 ml) sample volumes (Gochin *et al.*, 1981; Avgerinos *et al.*, 1991; Pokrajac *et al.*, 1998), involve a multi-step, lengthy extraction process or solid-phase extraction (DeAngelis *et al.*, 1990; Ronn *et al.*, 1999) or require the use of an ion-pairing agent for suitable chromatography with a C_{18} column (Laizure *et al.*, 1990). A large sample volume (e.g., 1 ml) is a problem in certain populations such as pediatric AIDS patients requiring trimethoprim therapy for PCP (Ronn *et al.*, 1999). Ronn, *et al.* improved the applicability to special populations by decreasing the sample volume

to 125 μL, but the sample preparation involved multiple steps including protein precipitation, transfer of supernatant and subsequent dilution with mobile phase (Ronn *et al.*, 1999). Therefore, the goal of this work was to establish a method that uses a small sample volume and an efficient, economical extraction method. In this paper, we introduce an HPLC method with simplified sample processing and ultraviolet wavelength detection to determine trimethoprim in human plasma. The method was used to determine steady-state trimethoprim plasma concentrations in healthy human subjects.

Experimental

Chemicals

Trimethoprim (≥ 98 %) reference standard and sulfamethazine (≥ 99%) (internal standard, IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Perchloric Acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Blank human plasma was purchased from the Central Blood Bank of Pittsburgh (Pittsburgh, PA, USA). Deionized water was obtained from an in-house Millipore (Billerica, MA, USA) water system.

HPLC Separation

The HPLC system consisted of a Waters 2695 separation module and a Waters 486 Tunable Absorbance Detector (Waters, Corp., Milford, MA, USA). Detection and quantification were performed using Millenium^{32} software version 3.1 (Waters, Corp., Milford, MA, USA). Separation was achieved with a Synergi® Polar-RP 4μ 150 mm x 4.6 mm column (Phenomenex, Torrance, CA, USA) heated to 30°C and an isocratic mobile phase of ammonium formate (pH

3.0; 50 mM)-acetonitrile (ACN)-methanol (MeOH) (90:6:4, v/v/v), delivered at a flow rate of 1 ml/min. The monitoring wavelength was set at 280 nm, with a run time of 14 minutes.

Standard Preparation

Trimethoprim stock solution was prepared at a concentration of 1 mg/ml in methanol. Dilutions prepared in methanol at concentrations of 250 and 50 μg/ml were used to prepare calibration standards and quality control (QC) samples. The internal standard (IS) sulfamethazine was dissolved in methanol to prepare a 50 μg/ml stock solution. Standards and QC samples were prepared at the beginning of the validation experiment by spiking batches of blank human plasma and dispensing into 200 μL aliquots. Aliquots were stored at –20°C until analysis was performed.

Plasma Sample Preparation

Aliquots of plasma (200 μL) were placed in microcentrifuge tubes, IS (10 μL or 500 ng) was added, and the tubes were briefly vortexed. Perchloric acid (25 μL) was added to each sample, which was then vortexed for 2 minutes, and centrifuged at 3000 g for 10 minutes. The supernatant was transferred to HPLC vials, and capped. A 75-μL aliquot was injected onto the HPLC system for analysis.

Calibration and Linearity

Calibration curves consisted of seven non-zero standard concentrations of trimethoprim in human plasma: 0.5, 1, 2.5, 5, 7.5, 10, 20 μg/ml. Duplicate calibration curves were assayed daily for 3 days, with the lowest concentration (0.5 μg/ml) prepared in triplicate. For each curve, the trimethoprim to IS peak height ratios were calculated and plotted against the nominal trimethoprim concentration. Calibration curves for trimethoprim were constructed by weighted ($1/y^2$) linear regression analysis.

Precision and Accuracy

Precision and accuracy were determined by the analysis of trimethoprim QC samples spiked at concentrations of 2, 8, and 16 μg/ml. Replicate QC samples (n = 12) were analyzed on Day 1 to determine intra-day precision and accuracy. Inter-day precision and accuracy was determined from replicate QC samples analyzed on Day 1 (n = 12), Day 2 (n = 6), and Day 3 (n = 6) for a total of 24 QC samples at each concentration. Mean, standard deviation and relative standard deviation (RSD) were calculated from the QC values and used in the estimation of intra- and inter-day precision. Accuracy (bias) is expressed as the percent deviation between the mean concentration relative to the nominal concentration.

Selectivity and Stability

Selectivity was analyzed by processing six different sources of blank plasma. Blank plasma samples were processed in duplicate and compared to plasma spiked with the lowest trimethoprim standard. Sample carryover was determined by inserting vials of blank mobile phase in various positions throughout the third validation run. Batches of high and low QC samples were prepared and subjected to three freeze-thaw cycles prior to processing and

analysis. After each freeze thaw cycle, aliquots were extracted and analyzed and determined acceptable at > 95% peak height of control samples not exposed to the freeze-thaw cycles. The stability of processed samples was determined by repeated analysis of high and low QC samples for over 24 hours post-extraction. The limit of quantitation (LOQ) was defined as the lowest standard value having a signal to noise (S:N) ratio of at least 10:1 and acceptable precision and accuracy (i.e., within 15%). The limit of detection (LOD) was defined as the smallest detectable peak having a S:N ratio of at least 3:1.

Extraction Efficiency

Extraction recovery of trimethoprim was calculated by comparing the response obtained from extracted QC samples versus the response observed after direct injection of reference samples. Reference samples consisted of water spiked with appropriate amounts of trimethoprim standards. Responses obtained from the reference samples were defined as 100%.

Application to Plasma Sampling

Trimethoprim pharmacokinetics were determined in eight healthy volunteers. The protocol was approved by the University of Pittsburgh Institutional Review Board and signed informed consent was obtained from each subject. Trimethoprim, 2 × 100 mg tablets (Watson Pharmaceuticals, Corona, California, USA), were administered every 12 hours for a total of 5 days. Multiple plasma samples were collected for 48 hours after reaching steady state on Day 4 and stored at –20°C until analyzed. Plasma trimethoprim concentrations were determined as described above.

Results

Chromatographic Separation

Representative chromatograms of plasma samples are shown in Figure 11. Figure 11A depicts a double-blank sample (no IS) and a sample spiked with trimethoprim 0.5 μg/ml (LOQ) and IS. Figure 11B shows a subject plasma sample obtained at steady-state during trimethoprim 200 mg twice daily administration. Retention times were approximately 7.1 and 12.2 minutes for trimethoprim and IS, respectively. The peaks of interest were well separated and free from interference with endogenous peaks. Sulfamethoxazole eluted at approximately 9 minutes (data not shown) and did not interfere with either peak of interest.

Precision, Linearity, and Accuracy

Linear calibration curves were obtained for trimethoprim over the concentration range of 0.5 to 20 μg/ml; the mean regression equation was: $Y = 0.2083 \pm 0.0013\ X + 0.0033 \pm 0.0002$. The correlation coefficients calculated for each run were >0.999. Response remained linear at all concentrations with no saturation of signal. Intra- and inter-day precision were within ± 5% and accuracy was within ± 6.25% (Table 9).

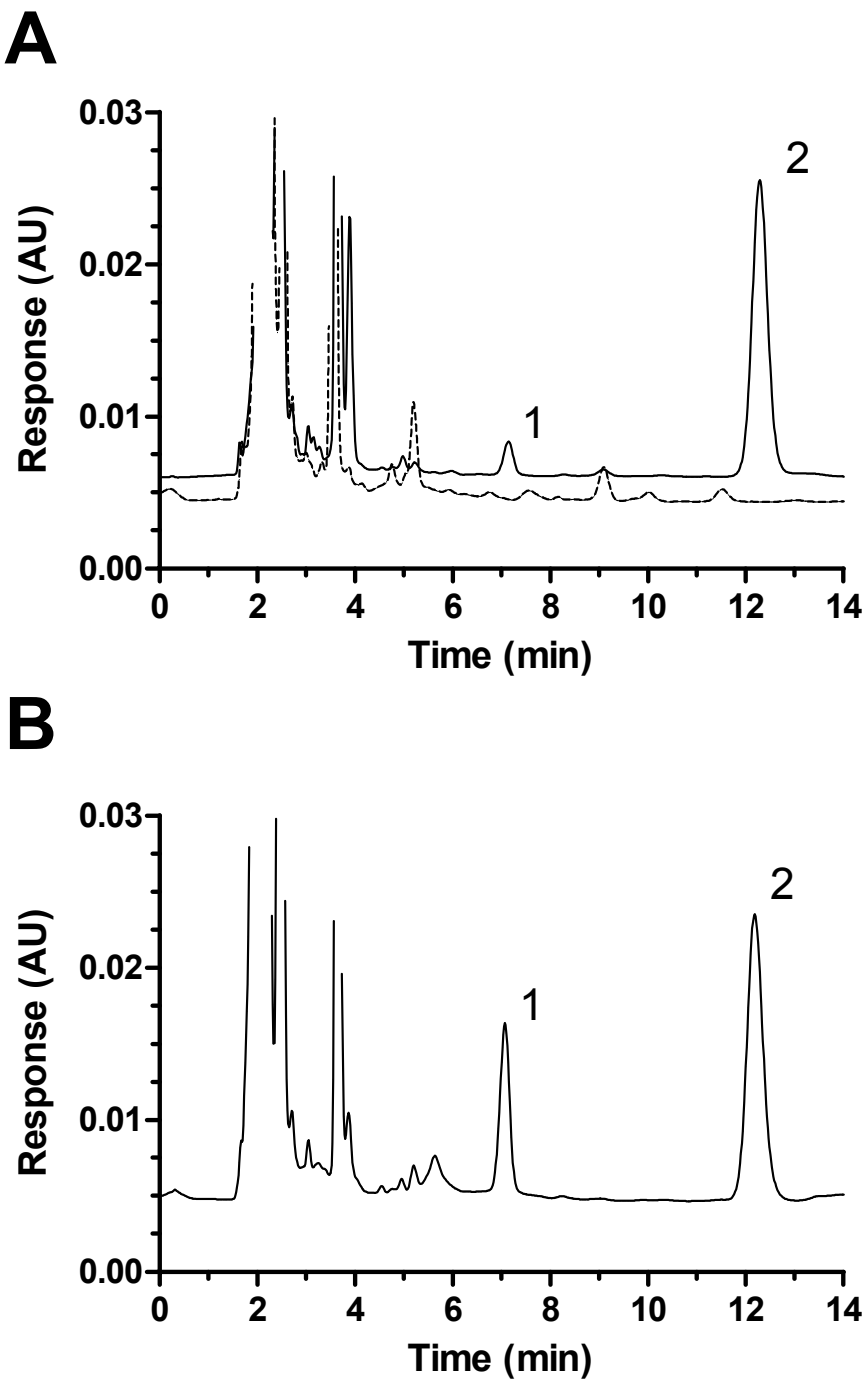


Figure 11 Representative chromatograms of (A) extracted double blank plasma (----) and plasma sample spiked (——) with trimethoprim 0.5 μg/ml (LOQ) and IS and (B) Subject plasma sample after administration of trimethoprim 200 mg twice daily at steady state (concentration of 2.89 μg/ml); Peaks are (1) Trimethoprim and (2) IS.

Table 9 Intra- and Inter-day precision and accuracy of trimethoprim in human plasma

	Concentration (μg/ml)		% RSD	% Deviation
	Spiked	Observed (mean ± S.D.)		
Intra-assay Precision				
Quality Controls				
	2.00	2.15 ± 0.04	1.7	7.5
	8.00	7.52 ± 0.14	1.9	-6.0
	16.0	15.3 ± 0.2	1.1	-4.4
Inter-assay Precision				
Quality Controls				
	2.00	2.12 ± 0.04	1.9	6.0
	8.00	7.64 ± 0.19	2.4	-4.5
	16.0	15.3 ± 0.4	2.7	-4.4
Standards				
	0.50	0.50 ± 0.01	1.4	-0.0
	1.00	1.01 ± 0.02	2.0	1.0
	2.50	2.55 ± 0.04	1.6	2.0
	5.00	4.99 ± 0.08	1.6	-0.2
	7.50	7.48 ± 0.07	0.9	-0.3
	10.0	10.2 ± 0.3	2.9	2.0
	20.0	19.5 ± 0.8	4.1	-3.0

Selectivity, Stability, and Recovery

No endogenous interference with trimethoprim or IS was observed in six extracted blank plasma samples and there was no evidence of sample carryover. The signal to noise ratio of trimethoprim at 0.5 μg/ml and IS at 50 μg/ml were greater than 10:1. The LOQ was 0.5 μg/ml and the LOD was 0.1 μg/ml with a S:N ratio of 5:1. Trimethoprim and the IS were stable in processed samples at room temperature for at least 24 hours prior to analysis. Samples subjected to three freeze-thaw cycles showed no sign of degradation. Extraction efficiency determined at the three QC concentrations was approximately 76 to 80% (Table 10).

Application to Plasma Sampling

Eight healthy subjects received trimethoprim 200 mg twice daily for 5 days. The mean (SD) steady-state concentration vs. time profile for all eight subjects on Day 4 is depicted in Figure 12. The concentrations observed were similar to those reported previously in HIV-infected subjects receiving trimethoprim 200 mg twice daily (Lee *et al.*, 1996).

Table 10 Extraction recovery of trimethoprim and internal standard in human plasma (n = 3)

Spiked Concentration	Recovery (%)	RSD (%)
Trimethoprim (μg/ml)		
2.0	80.5 ± 0.9	1.14
8.0	76.9 ± 0.7	0.96
16.0	79.6 ± 1.1	1.41
Internal Standard (μg/ml)		
50.0	90.7 ± 1.0	1.12

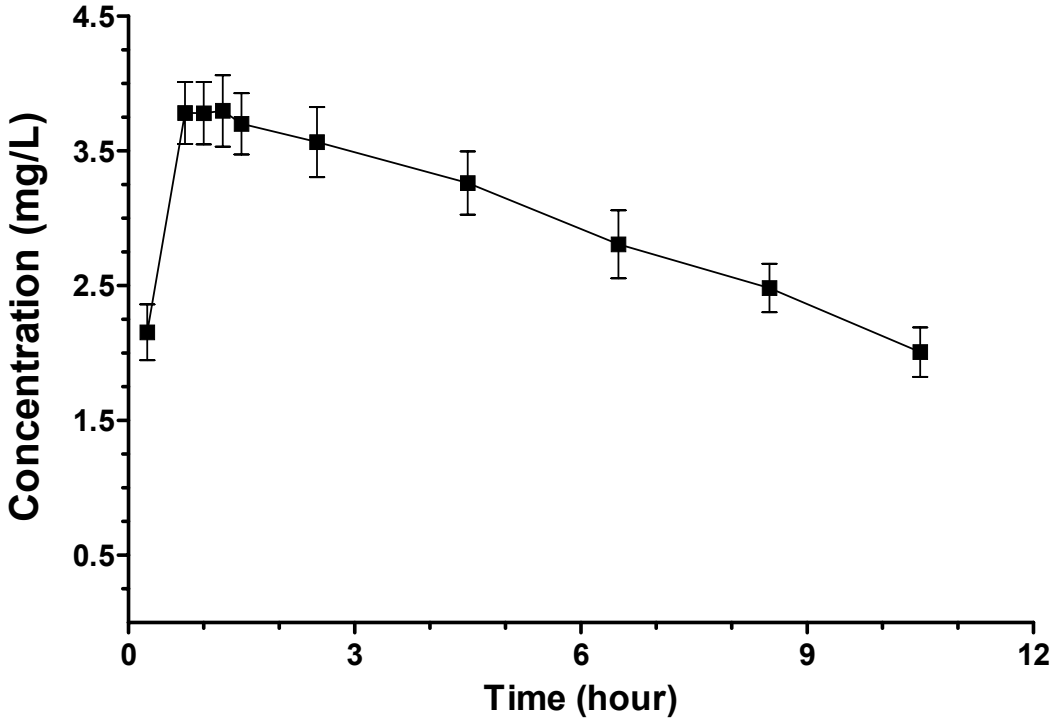


Figure 12 Mean ± SD concentration-time data at steady-state (Day 4) from 8 subjects after administration of trimethoprim 200 mg twice daily for 5 days.

Discussion

The method presented provides simplified and economical detection of trimethoprim in small volumes of human plasma, using protein precipitation coupled with ultraviolet detection. We used protein precipitation to extract trimethoprim, which is less time consuming than liquid-liquid extraction (Gochin *et al.*, 1981) and less expensive than solid phase extraction (DeAngelis *et al.*, 1990; Laizure *et al.*, 1990). Use of the phenyl Polar-RP column with a mobile phase containing a mixture of organic solvents yielded separation from endogenous interference and symmetrical peaks without the need for an ion-pairing agent. Further, sulfamethoxazole, an

agent commonly co-administered with trimethoprim, does not interfere with either peak of interest, so with modification this method could be used for pharmacokinetic studies involving the combination product. This method allows unbiased detection of trimethoprim and is directly applicable to trimethoprim steady-state pharmacokinetic studies.

Trimethoprim is most commonly administered in combination with sulfamethoxazole (e.g., Bactrim), but the single drug formulation is used for selected applications. Trimethoprim and sulfamethoxazole have been shown *in vitro* to selectively inhibit CYP2C8 and CYP2C9 activity, respectively; however, sulfamethoxazole loses selectivity and inhibits CYP2C8 activity at relatively high concentrations (Wen *et al.*, 2002). Therefore, to evaluate trimethoprim as a CYP2C8 inhibitor *in vivo*, the trimethoprim only formulation must be administered. The trimethoprim only formulation is also used in combination with dapsone as an alternative treatment for PCP in patients who can not tolerate sulfamethoxazole. Monitoring plasma concentrations of trimethoprim during PCP treatment may be helpful to verify therapeutic concentrations or to help avoid severe concentration-related adverse events, since increased neutropenia, thrombocytopenia, and severe adverse events has been associated with plasma concentrations greater than 8 μg/ml (Klinker *et al.*, 1998).

In summary, the method presented here uses a simple extraction method, has consistent recovery efficiency, and is applicable for analyzing trimethoprim plasma concentrations in steady-state pharmacokinetic studies or patients receiving high dose trimethoprim therapy.

Acknowledgements

This project was supported in part by NIH Research Grant R01 MH63458, funded by the National Institute of Mental Health and the Office of Dietary Supplements, and NIH/NCRR/GCRC#5M01RR00056.

Chapter 4

A Pyrosequencing Method for Genotyping Cytochrome P450 2C8 and 2C9 Enzymes

[Hruska MW, Frye RF, and Langaee TY. A Pyrosequencing Method for Genotyping Cytochrome P450 2C8 and 2C9 Enzymes. *Clin Chem* 2004;50(12):2392-5.]

Introduction

The cytochrome P450 enzymes CYP2C8 and CYP2C9 play important roles in drug metabolism (Dai *et al.*, 2001; Goldstein, 2001; Bahadur *et al.*, 2002). Single nucleotide polymorphisms that have been identified in the genes encoding for these enzymes may contribute to variability in drug response due to alterations in metabolism. CYP2C8 expression includes five alleles, *CYP2C8*1* (wild type), *CYP2C8*2*, *CYP2C8*3* (Dai *et al.*, 2001), *CYP2C8*4* (Bahadur *et al.*, 2002), and *CYP2C8*5* (Soyama *et al.*, 2001). The variant alleles *CYP2C8*2* and *CYP2C8*3* have been shown to have decreased enzyme activity *in vitro* (Dai *et al.*, 2001), while *CYP2C8*4* expression appears to cause decreased CYP2C8 activity (Bahadur *et al.*, 2002). The allele frequency of *CYP2C8*2* is 0.18 in the African-American population, while *CYP2C8*3* and *CYP2C8*4* are primarily expressed in the Caucasian population at frequencies of 0.13 and 0.075, respectively (Dai *et al.*, 2001; Bahadur *et al.*, 2002).

Thirteen alleles of CYP2C9 have been identified to date and the majority of studies on these have focused on the importance of *CYP2C9*2* (Crespi and Miller, 1997) and *CYP2C9*3* (Sullivan-Klose *et al.*, 1996). *CYP2C9*4* is expressed in the Asian population at an allele frequency of 0.02, but does not appear to be expressed in Caucasians or African-Americans (Imai *et al.*, 2000). *CYP2C9*5* and *CYP2C9*6* are found primarily in African Americans at frequencies of 0.017 (Dickmann *et al.*, 2001) and 0.006 (Kidd *et al.*, 2001), respectively. Blaisdell *et al.* (Blaisdell *et al.*, 2004) recently identified variants designated as *CYP2C9*7* through *CYP2C9*12*, which have allele frequencies ranging from 0.006 to 0.03 in African populations. The *CYP2C9*8*, *CYP2C9*11*, and *CYP2C9*12* alleles have altered *in vitro*

metabolic activities in comparison with the wild-type allele *CYP2C9*1* (Blaisdell *et al.*, 2004).. Finally, *CYP2C9*13* was identified in a Chinese population with an allele frequency of 0.01 and was associated with decreased CYP2C9 activity (Si *et al.*, 2004).

Given the importance of these enzymes in the metabolism of multiple drugs, a reliable genotyping method that is amenable to high throughput applications would help facilitate research in this area. Several restriction fragment length polymorphism (RFLP) methods have been developed to identify *CYP2C8* and *CYP2C9* alleles (Sullivan-Klose *et al.*, 1996; Crespi and Miller, 1997; Dai *et al.*, 2001; Bahadur *et al.*, 2002). These methods are reliable, but have several disadvantages that include the time required for enzymatic digestion, lack of feasible high throughput applications, potential for incomplete digestion, and increased cost per sample (Aquilante *et al.*, 2004). Other methods using the LightCycler (Burian *et al.*, 2002) and tetra-primer (Ye *et al.*, 2001) technologies offer advantages over RFLP, but do not provide researchers with direct sequencing data.

Pyrosequencing is a less laborious process that relies on the release of pyrophosphate upon incorporation of a nucleotide into a DNA sequence to provide direct sequencing data, is ideal for the determination of allelic variants and by utilizing Multiplex Pyrosequencing numerous target DNA sequences may be analyzed in a single tube (Ronaghi, 2001; Pourmand *et al.*, 2002). We previously developed a Pyrosequencing method for CYP2C9 genotyping, but the original method only determines the two major variants found in Caucasian and African-American populations, *CYP2C9*2* and *CYP2C9*3* (Aquilante *et al.*, 2004). An improvement to our previous reported method is necessary in order to evaluate *CYP2C9*4* and *CYP2C9*5*, which are located on the same exon as *CYP2C9*3*.

The purpose of this study is to introduce an analytical method that determines all of the major *CYP2C8* variants and the major *CYP2C9* variants expressed in the population, *CYP2C9*2*, and in a single reaction, *CYP2C9*3*, *CYP2C9*4*, and *CYP2C9*5*.

Methods

DNA extraction and isolation:

For DNA extraction and isolation, blood samples were collected from fifty consecutively identified healthy volunteers who signed informed consent. The study was approved by the Institutional Review Board at the University of Florida. Ethnicity was self-reported as African-American, 4 (0.08), Asian, 2 (0.04), Caucasian, 36 (0.72), and Hispanic, 8 (0.16). Approximately 10 ml of blood was withdrawn from each subject and DNA was extracted with a QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer instructions. Samples were stored at -20°C after extraction.

Primers and PCR Conditions:

Primers used for the PCR reactions and Pyrosequencing are shown in Table 11. PCR reactions (25 μL) consisted of 12.5 μL HotStarTaq™ Master Mix (Qiagen), 1.5 μL DMSO, PCR primers (10 pmol), 7 μL of H2O, and 40 ng DNA. PCR conditions were as follows: 95°C for 15 minutes, 40 cycles consisting of (1) denaturation at 94°C for 30 seconds, (2) annealing at 56°C (55°C for CYP2C9; 58°C for CYP2C8 Exon 8) for 30 seconds, and (3) extension at 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes.

Pyrosequencing:

Genotyping was performed using the previously described protocol (Aquilante *et al.*, 2004). Briefly, PCR products (10 μl) were immobilized with streptavidin-coated Sepharose beads. Beads were isolated and treated with 70% ethanol, denaturation buffer, and wash buffer and

released into a mixture of annealing buffer and 10 pmol sequencing primer (Table 11), heated at 80°C for 2 minutes, and cooled to room temperature. Pyrosequencing was performed for sequence determination and allele designation using a Biotage PSQ HS 96 System and data was captured on PSQ HS 96 SNP software. The sequence of ten random subjects was determined in triplicate to verify robustness of the assay.

Results

Figure 13(a) depicts the predicted histograms and subject pyrograms for CYP2C8 Exon 5 in subjects presenting with the following genotypes: (i) *CYP2C8*1/*1* , (ii) *CYP2C8*1/*2* , (iii) *CYP2C8*2/*2*, (iv) *CYP2C8*1/*4*, and (v) *CYP2C8*4/*4*. The predicted histogram and resultant pyrogram of a subject presenting as *CYP2C9*1/*3* is illustrated in Figure 13(b). The allelic frequencies of the sample population are listed in Table 11. There were no allelic variants found in the Asian subjects and *CYP2C9*4* and *CYP2C9*5* were not found in the population studied. Linkage disequilibrium was apparent between *CYP2C8*3* and *CYP2C9*2* as 7 of the 8 (87.5%) subjects expressing the *CYP2C8*3* allele also expressed *CYP2C9*2*. Re-analysis of ten randomly selected samples in triplicate produced the same genetic sequence information.

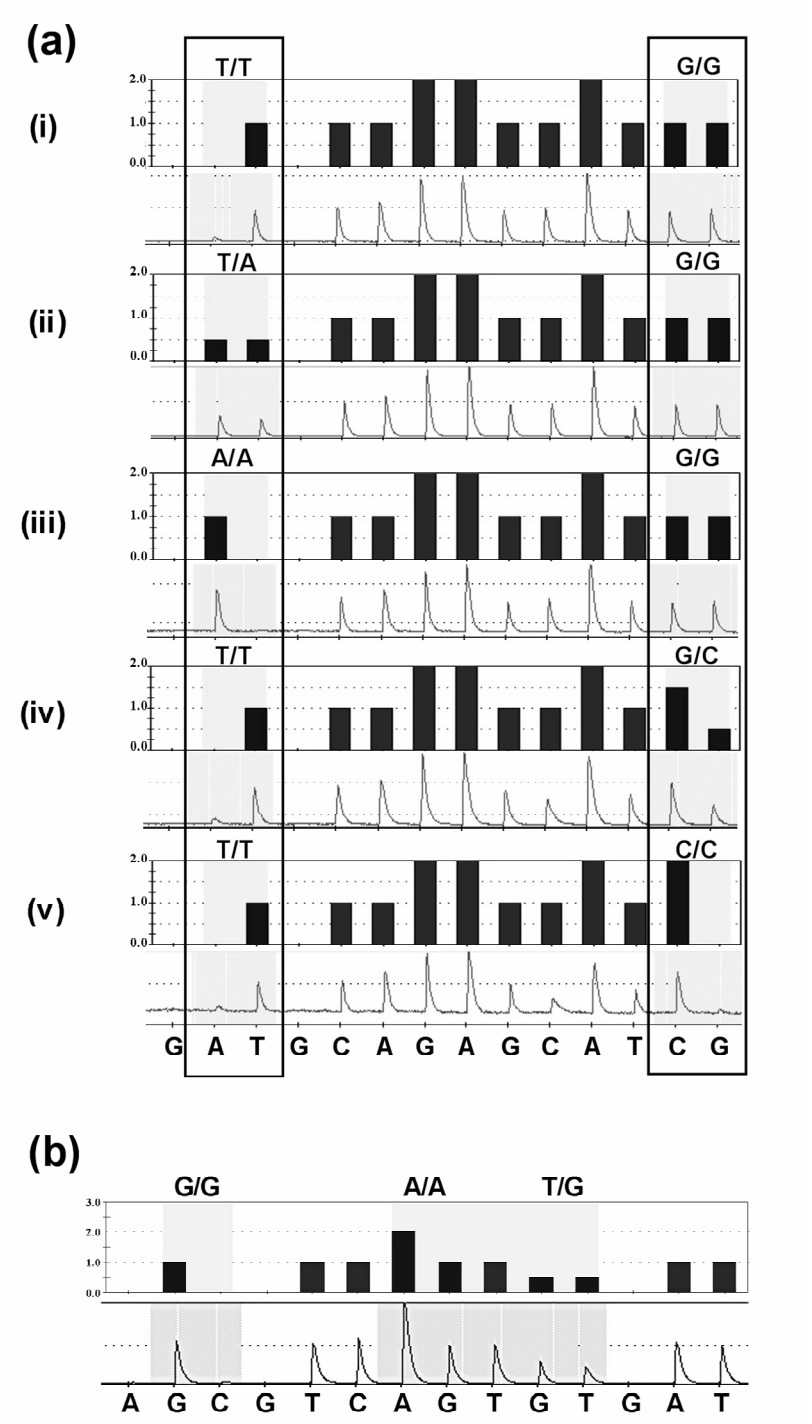


Figure 13 CYP2C8 Exon 5 sequence, predicted histograms and pyrograms.

(a) *CYP2C8* Exon 5 sequence used to determine genotypes for *CYP2C8*2* (805A>T, indicated by left box) and *CYP2C8*4* (792C>G, indicated by the right box). Beneath those are predicted histograms and pyrograms of subjects presenting as (i) homozygous wild type, *CYP2C8*1/*1* (A/A and C/C) (ii) *CYP2C8*1/*2* (A/T and C/C) (iii) *CYP2C8*2/*2* (T/T and C/C) (iv) *CYP2C8*1/*4* (A/A and C/G) and (v) *CYP2C8*4/*4* (A/A and G/G) genotypes. (b) Predicted histogram and pyrogram of a subject with *CYP2C9*1/*3* (A/C) genotype. Allelic variation of *CYP2C9* Exon 7 is determined from left to right as *CYP2C9*5* (1080C>G), *CYP2C9*4* (1076T>C), and *CYP2C9*3* (1075A>C). The order of nucleotide dispensation is located at the bottom of each pyrogram.

Table 11 PCR and Sequencing Primers and Allele Frequencies Observed for *CYP2C8*2*, *CYP2C8*3*, *CYP2C8*4*, and *CYP2C9*2*, *CYP2C9*3*, *CYP2C9*4*, and *CYP2C9*5* in 50 Subjects

Allele/ Exon	Nucleotide Change	PCR Primers	Sequencing Primer	Frequencies Published/ Observed
CYP2C8				
*2 5	805 A>T	F: **B**-5'-CAGGCTTGGTGTAAGATACATA-3'	5'-CTTACCTGCTCCATTTTGA-3'	0.18[(a)(b)]/ 0.04[(c)]
*4 5	792 C>G	R: 5'-CAGAAGGATTCGATGAATCACAA-3'		0.075[(d)] / 0.06
*3 3	416 G>A	F: **B**-5'-GTG TTCTCCCAGTTTCTGCCC-3' R: 5'-GACGCAGAGTAGAGTCACCCAC-3'	5'-CGGTCCTCATGCTC-3'	0.13[(b)] / 0.09
8	1196 A>G	F: 5'-CTCCTCACTTCTGGACTTCTTTA-3' R: **B**-5'-CCTTTAAATACAAATGGAAACGAG-3'	5'-CGTGCTACATGATGACA-3'	
CYP2C9				
*2 3	430 C>T	F: 5'-GTATTTTGGCCTGAAACCCATA-3'[(e)] R: **B**-5'-CACCCTTGGTTTTTCTCAACTC-3'[(e)]	5'-GGGAAGAGGAGCATTGAGGC-3'[(e)]	0.13[(f)(g)] / 0.09
*3 7	1075 A>C			0.08[(f)(g)] / 0.09
*4 7	1076 T>C	F: **B**-5'-TGCACGAGGTCCAGAGAT-3'[(e)] R: 5'- GATACTATGAATTTGGGACTTC-3'[(e)]	5'-GCTGGTGGGGAGAAG-3'[(e)]	0.02[(h)] / 0.00
*5 7	1080 C>G			0.017[(g)] / 0.00

Key: F: Forward; R: Reverse; **B**: Biotin labeled; (a) As reported in the African-American population (b) Observed in ref. (Dai *et al.*, 2001) (c) Only observed in African-American subjects (4 of 8 alleles) (d) Observed in ref. (Bahadur *et al.*, 2002) (e) As previously reported by Aquilante *et al.* (Aquilante *et al.*, 2004) (f) As reported in Caucasian populations (g) Observed in ref. (Dickmann *et al.*, 2001) (h) Observed in ref. (Imai *et al.*, 2000)

Discussion

The Pyrosequencing method presented here is directly applicable to studies involving genetic determination of *CYP2C8* and *CYP2C9* polymorphisms in a large sample population as our laboratory has used this assay to genotype several hundred samples (unpublished data) from a variety of sources including whole blood, urine, buccal swab, and mouthwash rinse. A key advantage of Pyrosequencing is that the direct sequence data for each subject DNA is obtained. By obtaining the DNA sequence, little interpretation is necessary versus other methods such as RFLP where incomplete digestion may complicate data analysis. Further, the time and cost per sample using Pyrosequencing is less than RFLP analysis (Aquilante *et al.*, 2004). A method using real-time PCR to determine *CYP2C8* allelic variants was recently reported (Weise *et al.*, 2004). The PCR method offers an advantage over RFLP in that it can be modified for high throughput applications, but it does not provide a direct sequence of DNA and therefore still requires some data interpretation. Further, this RT-PCR method does not involve the determination of CYP2C9 genotype, which is important as a linkage between the allelic variants *CYP2C8*3* and *CYP2C9*2* exists (Yasar *et al.*, 2002). The data gathered in our investigation supports the previous report as similar frequencies of *CYP2C8*3* and *CYP2C9*2* linkage were found, but further investigation with a larger sample is necessary. The method is also novel as it allows the genotyping of three separate allelic variants in one PCR reaction. Unfortunately, we were unable to detect *CYP2C9*4* and *CYP2C9*5* carriers as the respective Asian and African-American population sample sizes were small. A larger sample of these populations would be required to identify individuals carrying these alleles. Additionally, with the recent discovery of other *CYP2C9* allelic variants (Blaisdell *et al.*, 2004; Si *et al.*, 2004), adaptations of this method should allow detection of these variants.

Acknowledgment

This project was supported in part by NIH Research Grants R01 HL68834 and MH63458, funded by the National Institute of Mental Health and the Office of Dietary Supplements and NIH M01 RR00082.

Chapter 5

The Effect of Trimethoprim on CYP2C8 Mediated Rosiglitazone Metabolism in Human Liver Microsomes and Healthy Subjects

[Hruska MW, Amico JA, Langaee TY, Ferrell RE, Fitzgerald SM, and Frye RF. The Effect of Trimethoprim on CYP2C8 Mediated Rosiglitazone Metabolism in Human Liver Microsomes and Healthy Subjects. *Br J Clin Pharmacol* 2005;59(1):70-9.]

Abstract

Aims: Rosiglitazone, a thiazolidinedione antidiabetic medication used in the treatment of Type 2 diabetes mellitus, is predominantly metabolized by the cytochrome P450 (CYP) enzyme CYP2C8. The anti-infective drug trimethoprim has been shown *in vitro* to be a selective inhibitor of CYP2C8**.** The purpose of this study was to evaluate the effect of trimethoprim on the CYP2C8 mediated metabolism of rosiglitazone *in vivo* and *in vitro*.

Methods: The effect of trimethoprim on the metabolism of rosiglitazone *in vitro* was assessed in pooled human liver microsomes. The effect *in vivo* was determined by evaluating rosiglitazone pharmacokinetics in the presence and absence of trimethoprim. Eight healthy subjects (4 men and 4 women) completed a randomized, cross-over study. Subjects received single dose rosiglitazone (8 mg) in the presence and absence of trimethoprim 200 mg given twice daily for 5 days.

Results: Trimethoprim inhibited rosiglitazone metabolism both *in vitro* and *in vivo*. Inhibition of rosiglitazone *para*-hydroxylation by trimethoprim *in vitro* was found to be competitive with apparent K_i and IC_{50} values of 29 μM and 54.5 μM, respectively. In the presence of trimethoprim, rosiglitazone plasma AUC was increased by 31% (p = 0.01) from 2774 +/- 645 μg l(-1) hr to 3643 +/- 1051 μg l(-1) hr (95% confidence interval (CI) for difference 189, 1549), and half-life was increased by 27% (p = 0.006) from 3.3 +/- 0.5 to 4.2 +/- 0.8 hours (95% CI for difference 0.36, 1.5). Trimethoprim reduced the para-O-sulfate rosiglitazone/rosiglitazone and the *N*-desmethylrosiglitazone/rosiglitazone AUC(0-24) ratios by 22% and 38%, respectively.

Conclusions: These results indicate that trimethoprim is a competitive inhibitor of CYP2C8-mediated rosiglitazone metabolism *in vitro* and that trimethoprim administration increases plasma rosiglitazone concentrations in healthy subjects.

Introduction

The significance of cytochrome P450 (CYP) 2C8 to drug elimination is expanding due to its relative distribution, polymorphic expression, and the rapidly increasing knowledge of its role in drug metabolism. CYP2C8 is primarily distributed in the liver (Klose *et al.*, 1999), and it is the second most abundant member of the CYP2C subfamily expressed in this organ (Lapple *et al.*, 2003). CYP2C8 is also found in the lung (Mace *et al.*, 1998), kidney, adrenal gland, mammary gland, brain, uterus, and ovary tissue (Klose *et al.*, 1999). CYP2C8 is polymorphically expressed with five allelic variants identified to date, *CYP2C8*2* (Ile269Phe), *CYP2C8*3* (Arg139Lys, Lys399Arg) (Dai *et al.*, 2001), *CYP2C8*4* (Ile264Met) (Bahadur *et al.*, 2002), and *CYP2C8*5* (frame shift mutation) (Nakajima *et al.*, 2003). Altered *in vitro* activity has been demonstrated for the *CYP2C8*2* and *CYP2C8*3* variant alleles (Dai *et al.*, 2001; Bahadur *et al.*, 2002; Nakajima *et al.*, 2003). The *CYP2C8*2* allele is found mainly in African-Americans, has a frequency of 0.18, and is associated with decreased affinity for paclitaxel and a two-fold lower intrinsic clearance of 6α-hydroxypaclitaxel (due to a two-fold higher apparent Km with no change in Vmax). The *CYP2C8*3* allele has a frequency of 0.13, is found primarily in Caucasians, and is associated with decreased paclitaxel turnover (Dai *et al.*, 2001). These genetic polymorphisms may play a role in the pharmacokinetic and pharmacodynamic response to drugs metabolized by CYP2C8, which includes paclitaxel (Rahman *et al.*, 1994), amodiaquine (Li *et al.*, 2002), repaglinide (Bidstrup *et al.*, 2003), morphine (Projean *et al.*, 2003b), and rosiglitazone (Bolton *et al.*, 1996; Baldwin *et al.*, 1999; Cox *et al.*, 2000). In addition, CYP2C8 metabolizes retinoic acid (Nadin and Murray, 1999) and arachidonic acid (Zeldin *et al.*, 1995; Dai *et al.*, 2001) and so may have an important physiological role.

Rosiglitazone is a thiazolidinedione antihyperglycemic agent used in the treatment of type 2 diabetes (Baldwin *et al.*, 1999; Cox *et al.*, 2000). Thiazolidinediones exert their clinical effects via the peroxisome proliferator-activated receptor γ (PPAR γ), which facilitates the expression of genes responsible for glucose and lipid metabolism (Balfour and Plosker, 1999). Rosiglitazone undergoes extensive metabolism with essentially no parent drug excreted unchanged in the urine (Cox *et al.*, 2000). Rosiglitazone is primarily metabolized by CYP2C8, with CYP2C9 contributing to a minor extent (Baldwin *et al.*, 1999). The two major metabolites of rosiglitazone produced by CYP2C8 are *para*-hydroxyrosiglitazone and *N*-desmethylrosiglitazone (Bolton *et al.*, 1996; Baldwin *et al.*, 1999), which account for >80% of all metabolic products isolated in human plasma after rosiglitazone administration (Cox *et al.*, 2000). The sulfate conjugate of *para*-hydroxyrosiglitazone, *para*-O-sulfate-rosiglitazone is the major plasma metabolite (Cox *et al.*, 2000). Based on these characteristics, conversion of rosiglitazone to *para*-O-sulfate-rosiglitazone may be a useful index of CYP2C8 activity.

It has been suggested that one means by which an enzyme-selective probe drug can be validated is to evaluate its pharmacokinetics in subjects who are treated with an inhibitor of the target enzyme (Watkins, 1994). The nonspecific CYP2C8 and CYP2C9 inhibitor gemfibrozil has been shown to adversely affect the pharmacokinetics of rosiglitazone causing a 2.3-fold increase in the area under the concentration-time curve (Niemi *et al.*, 2003a). The effect of a selective CYP2C8 inhibitor on rosiglitazone pharmacokinetics has not been evaluated. The antibiotic trimethoprim, which is commonly used in the treatment of respiratory and urinary tract infections, has been shown *in vitro* to selectively inhibit CYP2C8 at concentrations ranging from 5 to 100 μM (K_i = 32 μM) (Wen *et al.*, 2002). Trimethoprim administered at a dose of 200 mg twice daily achieves peak plasma concentrations of approximately 20 μM (Watson *et al.*, 1983).

Based on the liver/plasma concentration ratio of 6.5 to 1 observed in monkeys (Craig and Kunin, 1973), it has been proposed that this plasma concentration would produce approximate hepatic concentrations of 130 μM, which would yield greater than 80% inhibition of CYP2C8 (Watson *et al.*, 1983; Wen *et al.*, 2002). Trimethoprim was recently shown to inhibit the metabolism of the CYP2C8 and CYP3A4 substrate repaglinide, causing a 61% increase in the area under the curve (Niemi *et al.*, 2004b). The effect of trimethoprim administration on the CYP2C8-mediated metabolism of rosiglitazone has not been determined. Therefore, the purpose of these studies was to evaluate the *in vitro* and *in vivo* inhibitory effect of trimethoprim on the metabolism of this drug.

Methods

Clinical Study

Human Subjects:

The study was approved by the Institutional Review Board at the University of Pittsburgh. Eight healthy non-smoking subjects (4 men and 4 women) provided written informed consent prior to the study. Subjects were determined to be healthy on the basis of past medical history, a physical examination and routine clinical laboratory tests. Subjects were excluded if they had any evidence of abnormal renal or hepatic function, had a BMI > 31 kg m^{-2}, had preexisting medical conditions, or used any medications other than oral contraceptives or hormone replacement therapy. Women of child bearing age were tested for pregnancy prior to enrollment and on admission to each study phase for exclusionary purposes. Subject age, weight, and body mass index were 28.3 ± 11.9 years (21 – 57), 72.2 ± 16.6 kg (50.9 – 101), and 23.6 ± 3.0 kg m^{-2} (19.5 – 28.0), respectively (Mean ± SD (range)). Seven subjects were Caucasian and one was

African-American. One of the female subjects was taking oral contraceptives and none of the subjects took complimentary medicine or over the counter medications during the study visits.

Study Design:

The study had a randomized cross-over design in which subjects received: 1) rosiglitazone 8 mg and 2) rosiglitazone 8 mg and trimethoprim 200 mg twice daily for five days. Study visits were separated by a washout period of 1 week. Subjects abstained from alcohol and caffeine containing foods and beverages for 24 hours, and from grapefruit or grapefruit juice and over-the-counter medications for 48 hours prior to each study visit. After an overnight fast, subjects were administered rosiglitazone 8 mg (Avandia®, GlaxoSmithKline, Research Triangle Park, NC, USA) with 8 oz (240 ml) of water at approximately 8:00 AM. Subjects fasted for approximately 2 hours after rosiglitazone dosing. Blood samples (10 ml) were obtained before and 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours after drug administration. In the other phase of the study, trimethoprim (200 mg) was administered for five days at approximately 7:30 AM and 7:30 PM. Rosiglitazone was administered at approximately 8:00 AM on the fourth day of trimethoprim dosing. Blood samples were obtained as above with additional samples collected at 13 hours prior to and 30, 36, and 48 hours after rosiglitazone administration. Blood samples were drawn in EDTA tubes, kept on ice, and centrifuged within two hours of collection at 2500 g, 4^{o}C, for 15 minutes. Plasma was harvested and stored at -70^{o}C until analysis.

Drug and Metabolite Analysis:

Rosiglitazone:

Concentrations of rosiglitazone were determined as described previously (Hruska and Frye, 2004b). Briefly, the internal standard, betaxolol (250 ng), was added to plasma (200 μl), and proteins were precipitated with 600 μl acetonitrile. Analysis was performed by HPLC with

fluorescence detection. Rosiglitazone was monitored at an excitation wavelength (λ_{ex}) of 247 nm and an emission wavelength (λ_{em}) of 367 nm and betaxolol was monitored at a λ_{ex} of 235 nm and a λ_{em} of 310 nm. Separation was achieved with an Alltima Phenyl 5µ 250 mm × 4.6 mm column (Alltech Associates Inc., Deerfield, IL, USA), and an isocratic mobile phase of 10 mM sodium acetate (pH 5): acetonitrile (60:40) delivered at a flow rate of 1 ml min^{-1}. Intra- and inter-day precision ranged from 3.1% – 8.5% and 2.3% – 5.7%, respectively.

***Rosiglitazone metabolites**:*

Plasma (100µl) was added to 96-well 0.45µ Captiva® filter plates (Varian Inc, Lake Forest, CA, USA) preloaded with 300 µl of acetonitrile. The plates were vortex-mixed for 30 seconds, inverted for 5 minutes, and filtered under a vacuum. Water (300 µL) was added to the filtrate, and plates were briefly vortex-mixed. Aliquots (10 µl) were injected onto a Surveyor HPLC system connected to a TSQ Quantum MS/MS system (Thermo, Woburn, MA, USA). Chromatographic separation was performed using a Symmetry C8 5µm, 2.1 × 150 mm column (Waters Corp., Milford, MA, USA). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol delivered at a flow rate of 200 µl min^{-1}. Gradient elution was achieved with mobile phase that started at 80% A, ramped to 40% A over 2 minutes, changed to 10% A from 2.5 to 6.0 minutes, and then ramped back to 80% A over the next 0.2 minutes. Conditions were held at 80:20 for 1.3 minutes for a total run time of 8.5 minutes. Detection was achieved with positive electrospray ionization and data output was captured with Xcalibur® software (Thermo, San Jose, CA, USA). Precursor and product ions $[M + H]^+$ detected were *m/z* 358.2/135, 374.2/151, and 344.2/121, for rosiglitazone, *para*-O-sulfate-rosiglitazone, and *N*-desmethylrosiglitazone, respectively (Bolton *et al.*, 1996; Cox *et al.*, 2000). Since the sulfate group is lost in the mass spectrometer source, the mass detected is that of the

para-hydroxy metabolite. The internal standard was detected with single ion monitoring $[M + H]^+$ at 308.3. Metabolite concentrations were calculated in arbitrary units (U l^{-1}) relative to the maximum peak concentration (C_{max}) determined from the chromatographic output.

Trimethoprim:

Concentrations of trimethoprim were determined as previously reported (Hruska and Frye, 2004a). Briefly, the internal standard sulfamethazine (500 ng) was added to plasma (200 µl) and proteins were precipitated with perchloric acid (25 µl). Samples were vortex-mixed and then centrifuged at 3000 *g* for 10 minutes. The supernatant was transferred to injector vials and an aliquot (75 µl) was injected onto the HPLC system. Separation was achieved using a Synergi® 4µ Polar-RP 150 mm × 4.6 mm column (Phenomenex, Torrance, CA, USA) heated to 30°C. The mobile phase, which consisted of ammonium formate (pH 3.0; 50 mM)-acetonitrile-methanol (90:6:4, v/v/v), was delivered isocratically at a flow rate of 1 ml min^{-1}. Detection was at an ultraviolet wavelength of 280 nm. Intra- and inter-day precision ranged from 1.1% – 1.7% and 1.9% – 2.7%, respectively.

***Determination of CYP2C8 Genotype*:**

Pyrosequencing assays were developed to genotype the *CYP2C8*2*, *CYP2C8*3*, and *CYP2C8*4* alleles. PCR reaction mixtures (25 µl) consisted of 12.5 µl HotStarTaq® Master Mix (Qiagen, Valencia, CA, USA), 1.5 µl DMSO, PCR primers (10 pmol each), 7 µl of water, and 40 ng DNA. PCR Primer sequences are shown in Table 12. PCR conditions were as follows: 95°C for 15 minutes, 40 cycles consisting of (1) denaturation at 94°C for 30 seconds, (2) annealing at 56°C (58°C for Exon 8) for 30 seconds, and (3) extension at 72°C for 45 seconds, followed by

final extension at 72°C for 7 minutes. Genotyping was performed using manufacturer protocol (Biotage AB, Uppsala, Sweden). Briefly, PCR products (10 μl) were immobilized with streptavidin-coated Sepharose beads and incubated. Beads were isolated and treated with 70% ethanol, denaturation buffer, and wash buffer. Beads were released into a mixture of annealing buffer and 10 pmol sequencing primer (Table 12), heated at 80°C for 2 minutes and cooled to room temperature (Ronaghi, 2001; Haglund *et al.*, 2004). Genotyping analysis was carried out using a PSQ HS 96 System (Biotage AB, Uppsala, Sweden) and data was captured with PSQ HS 96 SNP software (Biotage AB, Uppsala, Sweden).

Table 12 PCR Primers and Sequencing Primers for *CYP2C8*2*, *CYP2C8*3*, and *CYP2C8*4*

Exon	PCR Primers	Sequencing Primers
3	F: B-5'-GTGTTCTCCCAGTTTCTGCCC-3' R: 5'-GACGCAGAGTAGAGTCACCCAC-3'	5'-CGGTCCTCATGCTC-3'
5	F: B-5'-CAGGCTTGGTGTAAGATACATA-3' R: 5'-CAGAAGGATTCGATGAATCA CAA-3'	5'-CTTACCTGCTCCATTTTGA-3'
8	F: 5'-CTCCTCACTTCTGGACTTCTTTA-3' R: B-5'-CCTTTAAATACAAATGGAAACGAG-3'	5'-CGTGCTACATGATGACA-3'

Data Analysis:

The rosiglitazone concentration-time data were analyzed by noncompartmental methods. The terminal elimination rate constant (λz) was estimated by linear least squares regression analysis of the terminal portion of the log concentration-time data. Apparent elimination half-life was calculated from the expression $0.693\ \lambda z^{-1}$. The area under the concentration time curve (AUC) was determined using the linear trapezoidal rule with extrapolation to infinity. The apparent oral

volume of distribution (Vd/F) was calculated from the expression Dose $(\lambda z \times AUC)^{-1}$. The maximum concentration (C_{max}) was determined from the experimental data. T_{max} was the time at which C_{max} was observed. For both rosiglitazone metabolites, the area under the concentration time curve (AUC) was calculated by the linear trapezoidal rule from 0 to 24 hours. Pharmacokinetic calculations were performed using WinNonlin 2.1 (Pharsight Corp., Mountain View, CA, USA).

The trimethoprim concentration-time data were analyzed by non-compartmental methods. The AUC was calculated for the 48 hour period (four 12-hour dosing intervals) after rosiglitazone administration by the linear trapezoidal rule. The maximum steady state concentration (Css_{max}) and the minimum steady state concentration (Css_{min}) were those observed over all four dosing intervals. The average steady state concentration (Css_{ave}) was calculated by dividing the AUC(0-48) of trimethoprim by the total time (48 hours). All calculations were performed with WinNonlin 2.1 (Pharsight Corp., Mountain View, CA, USA).

Rosiglitazone pharmacokinetic parameters were log-transformed where appropriate and compared by paired t-test unless specified otherwise. Confidence intervals (95%) on mean differences were calculated. Tmax was compared using the Wilcoxon matched pairs test. A two-sided $p \leq 0.05$ was considered significant and all calculations were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Microsomal Inhibition Studies

Human Liver Microsomes:

Pooled human liver microsomes (n = 29 donors) were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA), who state that collection and processing of human tissue was conducted in compliance with all current regulatory and ethical requirements. Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI, USA) and gemfibrozil was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade or higher.

Incubation conditions:

Metabolite formation was linear with respect to time ($r^2 > 0.98$) and protein concentration ($r^2 > 0.97$). Optimal incubation conditions were 0.2 mg microsomal protein (0.8 mg ml^{-1}) incubated for 10 minutes at 37.0°C. Incubations were performed in duplicate and consisted of 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, and 0.2 mg of microsomal protein in 250 μl phosphate buffer pH = 7.4. Incubations were started by addition of glucose-6-phosphate dehydrogenase. Reactions were terminated by the addition of 400 μl of ice cold acetonitrile and samples were vortexed immediately, and centrifuged for 10 minutes at 3000 *g*. Supernatant (75 μl) was injected onto the column and analyzed as described above. The ratio of *para*-hydroxyrosiglitazone peak area to rosiglitazone peak area was used as a marker of CYP2C8 activity. The Km for the reaction was determined using substrate concentrations of 0, 5, 10, 25, 50, 75 and 100 μM.

Inhibition experiments:

To determine IC_{50}, rosiglitazone (10μM) was incubated in the presence of trimethoprim (0, 1, 5, 10, 25, 50, 100 μM) and gemfibrozil (0, 5, 10, 25, 50, 100, 250 μM). To determine K_i,

rosiglitazone was added to the microsomal suspension at varying concentration (5, 10, 25, 50 μM) in the presence of trimethoprim (0, 25, 50, 100 μM) and gemfibrozil (0, 25, 50, 100, 250 μM). Enzyme activity was plotted against nominal rosiglitazone concentration and the apparent Km was determined by fitting the Michaelis-Menten model to the untransformed data using non-linear regression. The IC_{50} and apparent inhibitory constant (K_i) values were determined by non-linear regression using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Different models of enzyme inhibition (i.e., competitive, noncompetitive, uncompetitive, and mixed-type inhibition) were fitted to the data and the best fit was determined by analysis of the residuals, the standard error and the 95% confidence interval of the parameter estimates, and the Akaike's information criterion.

Results

Rosiglitazone was well-tolerated and no adverse events were noted when the drug was given alone. One of the eight subjects developed a rash two days after completing trimethoprim therapy. The rash resolved without therapeutic intervention within two days.

Mean log concentration versus time profiles of eight subjects administered rosiglitazone in the presence and absence of trimethoprim are shown in Figure 14. Trimethoprim administration increased rosiglitazone AUC by 31% ($p = 0.01$) and $t_{1/2}$ by 27% ($p = 0.006$), but had no effect on C_{max}, t_{max}, and Vd/F (Table 13, Figure 15). There were no differences in *para*-O-sulfate-rosiglitazone or *N*-desmethylrosiglitazone AUC (Table 13), but the ratio of metabolite AUC to rosiglitazone AUC was decreased by 22% for *para*-O-sulfate-rosiglitazone ($p = 0.02$) and by 38% for *N*-desmethylrosiglitazone ($p = 0.004$) (See Appendix A, Figure 23). Trimethoprim administration decreased *N*-desmethylrosiglitazone C_{max} by 22% ($p = 0.02$) but did not significantly affect *para*-O-sulfate-rosiglitazone C_{max} ($p = 0.19$).

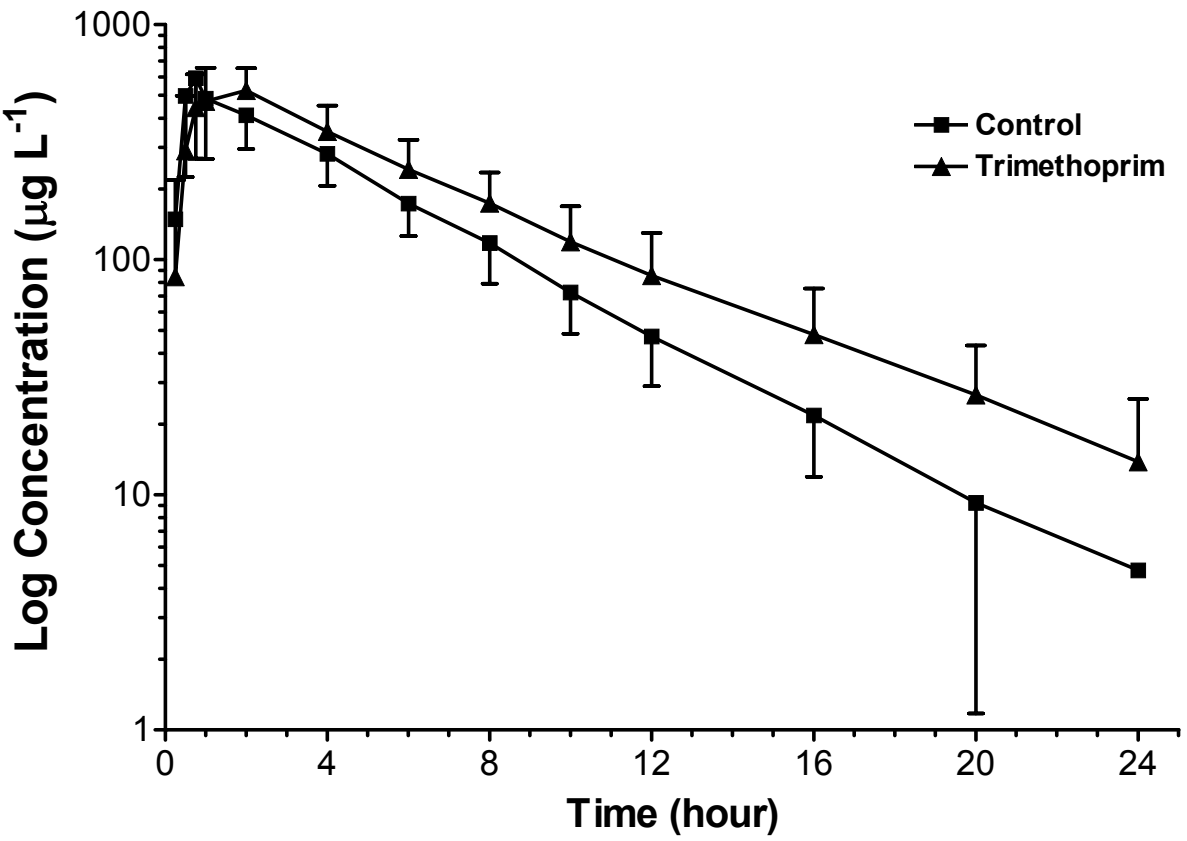


Figure 14 Log concentration versus time profile of rosiglitazone in the presence and absence of trimethoprim

Mean (± S.D.) log concentration versus time profile of rosiglitazone in the presence (*closed triangles*) and absence (*closed squares*) of trimethoprim, 200 mg given twice daily for five days. Data are from 8 healthy subjects.

Table 13 Mean pharmacokinetic parameters for rosiglitazone and metabolites in the presence and absence of trimethoprim, 200 mg given twice daily for five days to 8 healthy subjects.

Parameter	Control	Trimethoprim	Mean difference between control and trimethoprim (CI 95%)	P value
Rosiglitazone				
AUC (hr μg l^{-1})	2774 ± 645	3643 ± 1051	869 (189, 1549)	0.01
C_{max} (μg l^{-1})	674.3 ± 235.4	591.9 ± 62.2	-82.4 (-265.4, 100.7)	0.32
T_{max} (hr)	0.75 (0.5 – 4.0)	0.88 (0.5 – 2.0)		0.58
$t_{1/2}$ (hr)	3.3 ± 0.5	4.2 ± 0.8	0.9 (0.36, 1.5)	0.006
Vd/F (l)	14.9 ± 3.1	14.1 ± 2.9	-0.8 (-2.4, 0.75)	0.26
***Para*-O-sulfate-rosiglitazone**				
AUC (U hr l^{-1})	14.9 ± 3.0	14.2 ± 3.3	-0.61 (-2.4, 1.2)	0.45
AUC Ratio (POS: rosiglitazone)	5.5 ± 1.1	4.3 ± 1.7	-1.2 (-2.2, -0.3)	0.02
***N*-desmethylrosiglitazone**				
AUC (U hr l^{-1})	15.6 ± 2.4	13.1 ± 4.8	-2.5 (-5.9, 1.0)	0.14
AUC Ratio (NDR: rosiglitazone)	6.0 ± 1.7	3.7 ± 1.2	-2.2 (-3.5, -1.0)	0.004

Key: POS: *para*-O-sulfaterosiglitazone; NDR: *N*-desmethylrosiglitazone

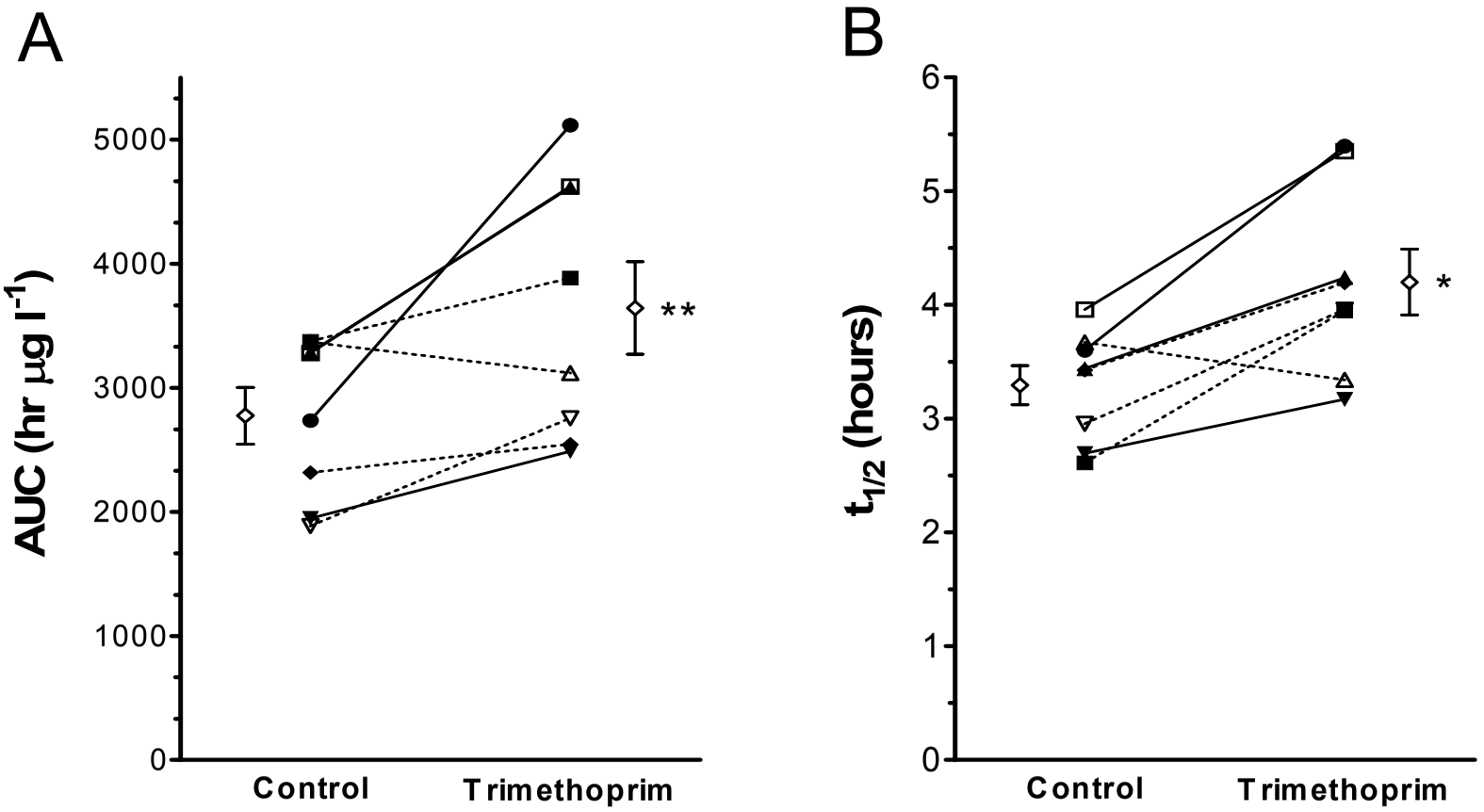


Figure 15 Rosiglitazone (A) AUC and (B) $t_{1/2}$ in the presence (Trimethoprim) and absence (Control) of trimethoprim, 200 mg given twice daily for five days to 8 healthy subjects. Solid lines indicate the wild type genotype (*CYP2C8*1/*1*) and dotted lines indicate the heterozygous genotype (*CYP2C8*1/*2* or *CYP2C8*1/*3*).

Subjects were genotyped as *CYP2C8*1/*1* (n=4), *CYP2C8*1/*2* (n=1) and *CYP2C8*1/*3* (n=3). Genotype did not appear to affect rosiglitazone metabolism, as there were no significant differences between subjects with heterozygous and homozygous wild type genotypes (See Appendix A, Figure 24). However, there was a trend for the fold increase in rosiglitazone AUC to be greater in wild type subjects versus subjects carrying an allelic variant (1.49 ± 0.26 vs. 1.16 ± 0.22, p = 0.1; See Appendix A, Figure 25).

Trimethoprim reached steady-state in all subjects with an observed mean Css_{ave} of 9.14 ± 1.83 μmol l^{-1}. The mean Css_{max} observed was 13.98 ± 2.43 μmol l^{-1} and the Css_{min} 6.78 ± 1.79 μmol l^{-1}. The mean AUC observed for each 12 hour dosing interval was 109.6 ± 24.6 μmol hr l^{-1}. There was a significant relationship between the Css_{ave} trimethoprim plasma concentration and the fold increase in rosiglitazone AUC in subjects having the *CYP2C8*1/*1* or **1/*2* genotype (r^2 = 0.97, p=0.0021; Figure 16). The relationship was not significant when subjects with the *CYP2C8*1/*3* genotype were included (r^2 = 0.08, p=0.48). There was no relationship between trimethoprim Css_{max} or Css_{min} values and the fold increase in rosiglitazone AUC.

In human liver microsomes, the apparent Km determined for rosiglitazone (mean (standard error)) was 11.9 μM (1.8). It should be noted that only one of the six substrate concentrations used in the incubations was below the estimated Km value. The inhibition profiles of rosiglitazone (10 μM) in the presence of trimethoprim and gemfibrozil at various concentrations are depicted in Figure 17. The apparent K_i and IC_{50} values calculated for trimethoprim were 29.0 μM (1.4) and 51.5 μM (2.8), respectively. The apparent K_i and IC_{50} values determined for gemfibrozil were 69.0 μM (1.1) and 119 μM (2.0), respectively. The mechanism of inhibition of CYP2C8 mediated metabolism of rosiglitazone was competitive and noncompetitive for trimethoprim and gemfibrozil, respectively (See Appendix A, Figure 26).

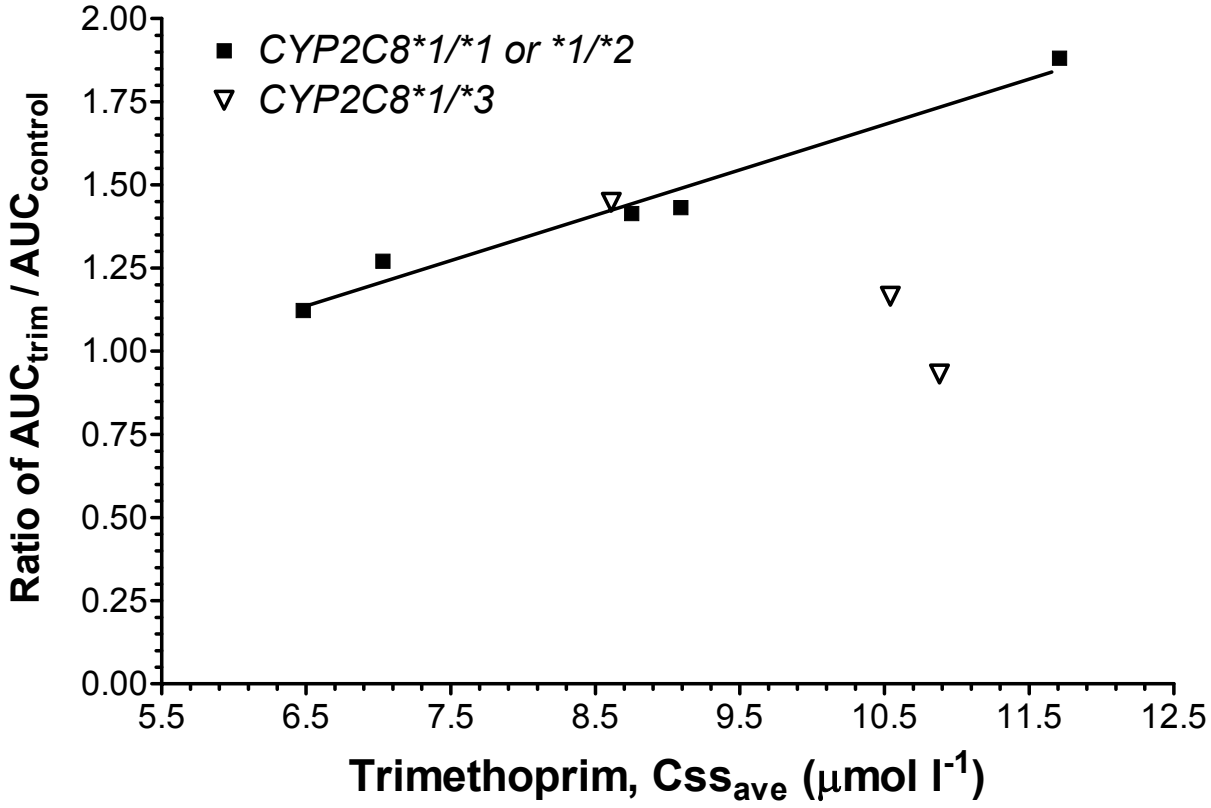


Figure 16 Relationship between the fold increase in rosiglitazone AUC and the trimethoprim Css_{ave} concentration. The linear regression line is based on data from subjects having the *CYP2C8*1/*1* or **1/*2* genotype (*closed squares*; $r^2 = 0.97$, p=0.0021). The relationship was not significant when subjects with the *CYP2C8*1/*3* genotype (*open triangles*) were included ($r^2 = 0.08$, p=0.48).

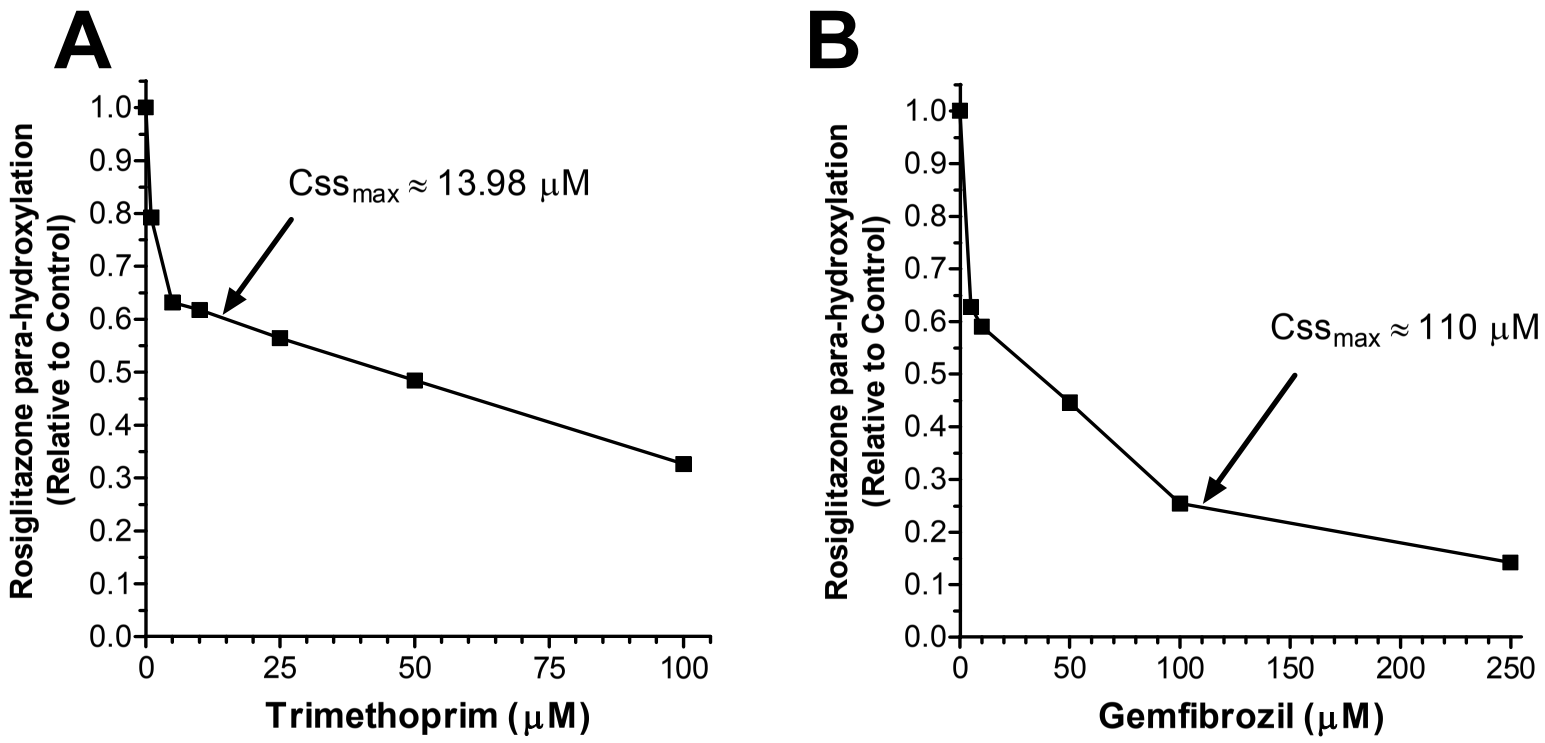


Figure 17 Rosiglitazone para-hydroxylation by human liver microsomes expressed as a percentage of control in the presence of (A) trimethoprim and (B) gemfibrozil. Arrows indicate (A) Css_{max} for trimethoprim administered 200 mg twice daily, which was determined in the present study, and (B) Css_{max} for gemfibrozil administered 600 mg twice daily, taken from reference, Niemi *et al.*, 2003a.

Discussion

Plasma rosiglitazone concentrations were increased in the presence of trimethoprim. Whereas the AUC and half-life were significantly increased, trimethoprim administration did not appear to affect rosiglitazone absorption, since t_{max}, C_{max}, and Vd/F were not different. *In vitro*, trimethoprim competitively inhibited the CYP2C8 catalyzed para-hydroxylation of rosiglitazone. Thus, the *in vitro* and *in vivo* results demonstrate that trimethoprim is an inhibitor of the CYP2C8 mediated metabolism of rosiglitazone.

The *in vitro* inhibition data for trimethoprim in the present study are consistent with previous reports (Wen *et al.*, 2002). Since gemfibrozil administration has been shown to increase rosiglitazone plasma concentrations in healthy human subjects (Niemi *et al.*, 2003a), the effect of gemfibrozil on rosiglitazone metabolism *in vitro* was also evaluated. Apparent K_i and IC_{50} values for gemfibrozil (69 μM and 119 μM) were comparable to the previously reported values of 69 μM and 91 μM, respectively (Wang *et al.*, 2002). These data indicate that trimethoprim is a more potent *in vitro* inhibitor of CYP2C8 compared to gemfibrozil, which suggests that trimethoprim may be more suitable as an *in vivo* inhibitor for this enzyme. However, gemfibrozil appears to be the more effective *in vivo* inhibitor of CYP2C8. Niemi *et al.*, found that the AUC of rosiglitazone after a single dose (4 mg) was increased 2.3 fold in the presence of gemfibrozil (600 mg given twice daily for three days) (Niemi *et al.*, 2003a). The difference in the magnitude of inhibition observed with trimethoprim and gemfibrozil may be due to the combined inhibition of CYP2C9 and CYP2C8 by the latter (Wen *et al.*, 2001; Wang *et al.*, 2002), although rosiglitazone is only metabolized by CYP2C9 to a minor extent (Baldwin *et al.*, 1999), or may be related to the plasma concentrations of the inhibitors. In the previous study, subjects achieved a mean peak total gemfibrozil concentration of 110 μM, which is

greater than the apparent K_i (69 μM), and a mean average total gemfibrozil concentration of 31 μM. Thus, based on total drug concentration, gemfibrozil would be expected to inhibit CYP2C8 (Niemi *et al.*, 2003a) to a greater extent than trimethoprim, since the maximum and average total concentrations of trimethoprim attained in plasma with normal therapeutic doses are less than the apparent K_i. Using the equation $AUC_I/AUC = 1+ [I]/ K_i$ (Ito *et al.*, 2004) and the apparent K_i (69 μM), the predicted fold-increase in rosiglitazone plasma AUC would be 2.6 and 1.5, based on peak and average total plasma gemfibrozil concentrations (inhibitor or I), respectively.

In the present study, the mean average total trimethoprim concentration (Css_{ave}) after administration for 5 days was 9.14 μM, which is similar to the previously reported value of 10.7 μM (Watson *et al.*, 1983). The mean peak total concentration was 13.98 μM, and this and Css_{ave} are less than the apparent K_i (29-32 μM). Wen *et al.*, based on the liver/plasma partition ratio of 6.5 to 1 observed in the rhesus monkey (Craig and Kunin, 1973) and an estimated peak total plasma concentration of 20 μM, predicted that subjects would achieve peak hepatic trimethoprim concentrations of approximately 130 μM, which would produce CYP2C8 inhibition of greater than 80% (Wen *et al.*, 2002). In the present study the rosiglitazone AUC was increased by only 1.3 fold. However, the predicted magnitude of CYP2C8 inhibition as expressed by the fold increase in rosiglitazone AUC would be 1.3 based on the average total plasma concentration. Factoring in estimated hepatic accumulation and plasma protein binding (trimethoprim fraction unbound=0.55), the predicted increase in AUC would be 2.7 or 2.1 fold based on peak or average unbound plasma concentration, respectively. Thus, these data suggest that hepatic accumulation during short term trimethoprim administration is not important and that the magnitude of inhibition is more closely related to the average total plasma concentration. Since the plasma concentrations of trimethoprim that are achieved with normal therapeutic doses are less than the

apparent K_i, the use of trimethoprim as an *in vivo* CYP2C8 inhibitor may be limited. Although gemfibrozil inhibits both CYP2C8 and CYP2C9 *in vitro* (Wen *et al.*, 2001; Wang *et al.*, 2002), it may still have utility as a selective CYP2C8 inhibitor, since *in vivo* studies have demonstrated a more pronounced effect on the metabolism of CYP2C8 substrates (e.g., cerivastatin, repaglinide, and rosiglitazone) compared to CYP2C9 substrates (e.g., glimepiride) (Niemi *et al.*, 2001b; Backman *et al.*, 2002; Niemi *et al.*, 2003b).

The trimethoprim-rosiglitazone interaction may have clinical relevance. The most common serious adverse effects associated with the thiazolidinediones rosiglitazone and pioglitazone are related to volume expansion (e.g., congestive heart failure, pulmonary oedema, and pleural effusions) (Niemeyer and Janney, 2002), which appears to be concentration-dependent (Idris *et al.*, 2003). Thus, patients taking rosiglitazone who are then treated with trimethoprim may be at greater risk of adverse effects such as oedema. The dosage of trimethoprim used in this study (200 mg twice daily for 5 days) is similar to the usual dosage of trimethoprim (160 mg twice daily) administered in combination with sulfamethoxazole for the treatment of common infections. Since the administration of trimethoprim with rosiglitazone may increase the risk of adverse events, the former should be used with caution, especially in patients treated with a high rosiglitazone dose (e.g., 8 mg daily), or who will receive trimethoprim for a prolonged period of time.

Although the sample size in this study is small, *CYP2C8* genotype did not appear to affect the metabolism of rosiglitazone (in the absence of trimethoprim), as there was no significant difference in rosiglitazone exposure in the subjects with a variant allele compared to the wild-type subjects (Figure 15A, control). However, the data suggest that the magnitude of inhibition by trimethoprim may be influenced by genotype, as wild type subjects tended to have

a greater increase in rosiglitazone AUC relative to control compared to subjects carrying either the *CYP2C8*2* or *CYP2C8*3* allele (See Appendix A, Figure 25). In addition, there was a strong correlation between the magnitude of inhibition, expressed as the fold increase in rosiglitazone AUC, and the average trimethoprim plasma concentration (Css_{ave}), but only in the subjects having the *CYP2C8*1/*1* or **1/*2* genotype (Figure 16). Differences in activity associated with the *CYP2C8*2* and *CYP2C8*3* alleles may contribute to these observations. *In vitro*, the *CYP2C8*2* polymorphism is associated with decreased affinity (increased Km) for paclitaxel with no change in Vmax, resulting in a two-fold lower 6α-hydroxypaclitaxel intrinsic clearance. The *CYP2C8*3* variant is associated with markedly decreased paclitaxel turnover (Dai *et al.*, 2001). Niemi *et al.*, recently evaluated the effect of *CYP2C8* genotype on the pharmacokinetics of the CYP2C8 and CYP3A4 substrate repaglinide. They found that subjects possessing the *CYP2C8*3* allele had lower repaglinide exposure compared to the control group, which suggests that the *CYP2C8*3* allele may result in greater CYP2C8 activity (Niemi *et al.*, 2003c). Data from this study suggest that genotype may influence the ability of trimethoprim to inhibit CYP2C8 and those individuals carrying the *CYP2C8*3* allele may be less sensitive to the inhibitory effects of trimethoprim on rosiglitazone metabolism.

Overall, this study demonstrates that trimethoprim competitively inhibits the CYP2C8 mediated metabolism of rosiglitazone *in vitro* and significantly increases rosiglitazone exposure in healthy subjects during short term administration. Therefore, trimethoprim should be used with caution in patients with type 2 diabetes who are taking rosiglitazone.

Acknowledgements: This project was supported in part by NIH Research Grant R01 MH63458, funded by the National Institute of Mental Health and the Office of Dietary Supplements and NIH/NCRR/GCRC#5M01RR00056.

Chapter 6

Effect of St. John's Wort Administration on Rosiglitazone Pharmacokinetics in Relation to *CYP2C8* genotype.

[Hruska MW, Cheong JA, Langaee TY, and Frye RF. Effect of St. John's wort Administration on Rosiglitazone Pharmacokinetics in Relation to *CYP2C8* genotype. *Submitted*.]

Abstract

Objective: The objectives of this study were (1) to investigate the effect of St. John's wort on rosiglitazone pharmacokinetics and (2) to evaluate whether the effect is influenced by CYP2C8 genotype.

Methods: This fixed sequence design study involved twenty-seven subjects sequentially selected with the following CYP2C8 genotypes: *CYP2C8*1/*1* (n = 8), *CYP2C8*1/*2* (n = 4), *CYP2C8*1/*3* (n = 7), *CYP2C8*1/*4* (n = 6), *CYP2C8*2/*2* (n = 1), and *CYP2C8*3/*3* (n = 1). The pharmacokinetics of rosiglitazone (8 mg) was evaluated after single dose administration in the absence and presence of St. John's wort 900 mg daily for fourteen days.

Results: St. John's wort administration induced the CYP2C8 mediated metabolism of rosiglitazone as the area under the plasma concentration-time curve (AUC) decreased by 26%, from 3190 ± 641 μg*hr/L to 2375 +/- 537 μg*hr/L ($p < 0.0001$, 90% Confidence Interval [CI], 71% - 78%) and apparent oral clearance (CL/F) increased by 35 % from 2.6 ± 0.6 L/hr to 3.5 +/- 0.7 L/hr ($p < 0.0001$, 90% CI, 128% - 142%). *CYP2C8* genotype had no effect on induction by St. John's wort as the rosiglitazone AUC ratio (St. John's Wort/control, %) was not significantly different between *CYP2C8*1/*1* , 71% (90% CI, 60% - 83%) and heterozygous carriers of *CYP2C8*2*, 78% (90% CI, 69% - 88%), *CYP2C8*3*, 70% (90% CI, 62% - 79%), and *CYP2C8*4*, 75% (90% CI, 67% - 84%).

Conclusions: Administration of St. John's wort significantly increases the CYP2C8 mediated clearance of rosiglitazone after single dose administration, but the magnitude of induction is not influenced by CYP2C8 genotype. St. John's wort use should be monitored when patients are administered rosiglitazone or other CYP2C8 substrates.

Introduction

Rosiglitazone is used in the treatment of type 2 diabetes and belongs to the thiazolidinedione class of antihyperglycemic agents (Balfour and Plosker, 1999; Cox *et al.*, 2000). These agents exert their clinical effects via the peroxisome proliferator-activated receptor γ (PPARγ), by facilitating the expression of genes responsible for glucose and lipid metabolism (Balfour and Plosker, 1999; Vamecq and Latruffe, 1999). Rosiglitazone is primarily metabolized by Cytochrome P450 (CYP) 2C8 with a minor contribution by CYP2C9 (Baldwin *et al.*, 1999). Rosiglitazone is metabolized extensively with essentially no parent drug excreted unchanged in the urine. The two major metabolites produced are *para*-O-sulfate-rosiglitazone and N-desmethyl rosiglitazone, which account for > 80% of all metabolic products isolated in human plasma (Cox *et al.*, 2000). To date, rosiglitazone pharmacokinetics have been shown to be modified by the CYP2C8 inhibitors gemfibrozil (Niemi *et al.*, 2003a) and trimethoprim (Niemi *et al.*, 2004a; Hruska *et al.*, 2005), as well as the classical CYP inducer, rifampin (Niemi *et al.*, 2004a; Park *et al.*, 2004a).

Rifampin and hyperforin, a primary constituent of the herbal remedy St. John's wort, have been shown *in vitro* to induce CYP enzymes (e.g., CYP3A4 and CYP2C9) through interaction with the pregnane X receptor (PXR) (Moore *et al.*, 2000; Wentworth *et al.*, 2000; Gerbal-Chaloin *et al.*, 2001; Rae *et al.*, 2001; Raucy *et al.*, 2002). Induction of CYP transcription by PXR begins when a ligand binds to PXR, causing a translocation of the ligand-PXR complex into the nucleus and the heterodimerization with the retinoic X receptor α (RXRα). The PXR-RXRα heterodimer binds to the xenobiotic response element initiating transcription and CYP enzyme production (Rae *et al.*, 2001). *In vivo*, rifampin and St. John's wort have been shown to increase the clearance of multiple drugs including *S*-mephenytoin

(CYP2C19) (Feng *et al.*, 1998; Wang *et al.*, 2004b), *S*-warfarin (CYP2C9) (Heimark *et al.*, 1987; Jiang *et al.*, 2004), and midazolam (CYP3A) (Backman *et al.*, 1996; Wang *et al.*, 2001). Collectively, these data demonstrate that St. John's wort (hyperforin) is an important modulator of CYP enzyme activity. The effect of St. John's wort on *in vivo* CYP2C8 activity is not known, but may be relevant since PXR activators (e.g., rifampin) have been shown to induce CYP2C8 expression in human hepatocytes (Gerbal-Chaloin *et al.*, 2001; Rae *et al.*, 2001; Raucy *et al.*, 2002).

Although not studied extensively, there is evidence to support that the magnitude of a drug interaction may be influenced by the CYP genotype. For example, induction of CYP2C19 activity by St. John's wort and rifampin is markedly different in extensive metabolizers (EM) and poor metabolizers (PM) (Feng *et al.*, 1998; Wang *et al.*, 2004a; Wang *et al.*, 2004b). St. John's wort administration causes an increase in the urinary excretion of 4′-hydroxymephenytoin (151%) and 5′-hydroxyomeprazole AUC (50%) in EMs, but has no effect on these parameters in PMs, which would be expected since PMs do not have a functional copy of the *CYP2C19* gene (Wang *et al.*, 2004b). Also, the effect of rifampin on mephenytoin metabolism is much greater in homozygous as compared to heterozygous EMs (Feng *et al.*, 1998). Consequently, it may be important to evaluate drug interactions in association with genotype. CYP2C8 has at least two functionally relevant polymorphisms *in vitro*: *CYP2C8*2* and *CYP2C8*3* (Dai *et al.*, 2001). The *CYP2C8*2* polymorphism encoding an Ile269Phe substitution is associated with a two-fold lower intrinsic clearance of paclitaxel and a decreased affinity for 6α-hydroxypaclitaxel, a major metabolite of paclitaxel; whereas *CYP2C8*3*, which encodes Arg139Lys and Lys399Arg substitutions, is associated with decreased paclitaxel turnover (Dai *et al.*, 2001). Allelic frequencies have been estimated as 0.18 for *CYP2C8*2* in African-Americans and 0.13 for

*CYP2C8*3* in Caucasians (Dai *et al.*, 2001). A third polymorphism, *CYP2C8*4*, which encodes an Ile264Met substitution and has an allelic frequency of 7.5% in the Caucasian population, may also have functional significance (Bahadur *et al.*, 2002). A previous study conducted by our laboratory demonstrated that *CYP2C8* genotype may contribute to the ability of the selective inhibitor trimethoprim to modulate CYP2C8 activity. In that study, there was a strong correlation between the fold-increase in rosiglitazone AUC and the average trimethoprim plasma concentration, but only in the subjects having the *CYP2C8*1/*1* or **1/*2* genotype (Hruska *et al.*, 2005). Therefore, it would be of interest to determine whether genotype modifies the extent to which the CYP inducer St. John's wort may alter CYP2C8 activity. Thus, the purpose of this study is to evaluate the effect of St. John's wort on CYP2C8 activity and to assess the impact of *CYP2C8* genetic variability on any change in CYP2C8 activity as measured by rosiglitazone pharmacokinetics.

Methods

Human Subjects:

This study was approved by the Institutional Review Board at the University of Florida. One hundred eleven healthy, non-smoking subjects (47 male, 64 female), 18 years of age or older provided written informed consent to participate. Subjects self-reported their racial background and past medical history, and were screened on the basis of *CYP2C8* genotype. Subjects were enrolled into genotype groups with a maximum of eight subjects (four men and four women) per group. Subjects asked to continue in the experimental phase of the study were determined to be healthy on the basis of past medical history, a physical examination and routine clinical laboratory tests. Subjects were excluded if they had any evidence of abnormal renal or hepatic function, had a BMI > 31 kg m^{-2}, had preexisting medical conditions, or used dietary/herbal

supplements or any medications other than oral contraceptives. Women of child bearing age were tested for pregnancy prior to enrollment and on admission to each study phase for exclusionary purposes.

Study Design:

This study was a fixed sequence design and subjects were stratified based on their *CYP2C8* genotype into one of 4 groups, *CYP2C8*1*, *CYP2C8*2*, *CYP2C8*3*, or *CYP2C8*4*. Subjects were administered rosiglitazone as follows: (1) rosiglitazone 8 mg alone, and (2) rosiglitazone 8 mg during St. John's wort 300 mg three times daily. Subjects abstained from alcohol, caffeine containing foods and beverages, grapefruit or grapefruit juice, and over-the-counter medications for 48 hours prior to each study visit. After an overnight fast, subjects were administered rosiglitazone 8 mg (Avandia®, GlaxoSmithKline, Research Triangle Park, NC, USA) with 8 oz (240 ml) of water at approximately 8:00 AM on study day 1. Subjects fasted for approximately 2 hours after rosiglitazone dosing. Blood samples (10 ml) were obtained before and 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours after drug administration. After the 24 hour blood sample was obtained on study day 2, subjects began self-administering St. John's wort extract 300 mg (Kira, "LI 160", Lichtwer Pharma AG, Berlin, Germany), three times daily for 14 days. Rosiglitazone was administered at approximately 8:00 AM on the fourteenth day of St. John's wort dosing (study day 15), and blood samples were obtained as above. Blood samples were drawn in EDTA tubes, kept on ice, and centrifuged within two hours of collection at 2500 g, 4^{o}C, for 15 minutes. Plasma was harvested and stored at -70^{o}C until analysis. Urine was collected from 0 to 24 hours after rosiglitazone administration and aliquots were stored at -20°C or colder until analyzed.

Sample Analysis:

Rosiglitazone:

Rosiglitazone plasma samples were analyzed as described previously (Hruska and Frye, 2004b). Briefly, 200 μL of plasma was placed into microcentrifuge tubes, 250 ng of betaxolol (internal standard) was added, and briefly vortexed. Acetonitrile (600 μl) was added to precipitate proteins and samples were vortexed and centrifuged at 3000 g for 10 minutes. Supernatant was transferred and evaporated to dryness at 45°C under a stream of nitrogen and reconstituted in 200 μl of mobile phase. Analytes were monitored using HPLC linked with fluorescence detection. Monitoring conditions for rosiglitazone were excitation wavelength (λ_{ex}) of 247 nm and an emission wavelength (λ_{em}) of 367 nm and an λ_{ex} of 235 nm and an λ_{em} of 310 nm for betaxolol. Separation was achieved with an Alltima Phenyl 5μ 250 mm × 4.6 mm column (Alltech Associates Inc., Deerfield, IL, USA), and an isocratic mobile phase of 10 mM sodium acetate (pH 5): acetonitrile (60:40) delivered at a flow rate of 1 ml min^{-1}. Intra- and inter-day precision ranged from 3.1% – 8.5% and 2.3% – 5.7%, respectively.

Cortisol and 6β-hydroxycortisol:

Urinary concentrations of cortisol and 6β-hydroxycortisol were determined by liquid chromatography-tandem mass spectrometry (Ohno *et al.*, 2000). Urine samples (1 ml) were extracted with ethyl acetate (5 ml) after the addition of the internal standard, fluorocortisone (1000 ng). The organic layer was transferred to a clean test tube and evaporated to dryness at 45°C under a stream of nitrogen gas. Samples were reconstituted with 200 μl of methanol:water (70:30, v/v) and placed into autosampler vials for injection. Separation was achieved using a Symmetry C18 5 μm, 3.0 x 150 nm, column (Waters Corporation, Milford, MA, USA). The mobile phases consisted of (A) water:methanol (80:20, v/v) and (B) methanol and were delivered

in a gradient with a total flow rate of 500 µl/min. The gradient was as follows: 100:0 (A:B) for 0.2 minutes, increased to 45:55 over 1.0 minutes, increased to 10:90 over 3.8 minutes, maintained at 10:90 for 0.5 minute, decreased to 100:0 over 0.5 minutes, and held at 100:0 for 1.5 minutes for a total of 7.5 minutes. Analytes were detected by tandem-mass spectrometry with positive-ion atmospheric pressure chemical ionization (TSQ Quantum Triple Quadrupole Mass Spectrometer, Thermo Electron, San Jose, CA, USA). Precursor [M + H]+ and product ions detected were 363.3/327.1, 379.3/325.1, and 423.3/239.1 for cortisol, 6β-hydroxycortisol, and fluorocortisone, respectively. Inter and intra day variability for both cortisol and 6β-hydroxycortisol were less than 5%.

Determination of CYP2C8 Genotype:

CYP2C8 genotype was determined as previously described (Hruska *et al.*, 2004) (Chapter 4). Briefly, a blood sample (approximately 10 ml) was withdrawn from each study subject and DNA was extracted using QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer protocol. PCR reaction mixtures (25 µl) consisted of 12.5 µl HotStarTaq® Master Mix (Qiagen, Valencia, CA, USA), 1.5 µl DMSO, PCR primers (10 pmol each), 7 µl of water, and 40 ng DNA. PCR conditions were as follows: 95°C for 15 minutes, 40 cycles consisting of (1) denaturation at 94°C for 30 seconds, (2) annealing at 56°C (58°C for Exon 8) for 30 seconds, and (3) extension at 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes. Genotyping was performed using manufacturer protocol (Biotage AB, Uppsala, Sweden). Briefly, PCR products (10 µl) were immobilized with streptavidin-coated Sepharose beads, incubated at room temperature, and were isolated and treated with 70% ethanol, denaturation buffer, and wash buffer. Beads were released into a mixture of annealing buffer and 10 pmol sequencing primer, heated at 80°C for 2 minutes and cooled to room temperature. Genotyping

analysis was carried out using a PSQ HS 96 System (Biotage AB, Uppsala, Sweden) and data was captured with PSQ HS 96 SNP software (Biotage AB, Uppsala, Sweden).

Data Analysis

Rosiglitazone Pharmacokinetics:

The rosiglitazone concentration-time data were analyzed using noncompartmental methods. The terminal elimination rate constant (λz) was estimated by nonlinear least squares regression analysis of the terminal portion of the concentration-time data. Apparent elimination half-life was calculated as 0.693/λz. The area under the concentration time curve (AUC) was calculated by the linear trapezoidal rule with extrapolation to infinity. The Cmax and time to reach Cmax (tmax) were determined by visual inspection of the plasma concentration versus time curves. Apparent oral clearance (CL/F) was determined as [Dose / $AUC_{0-\infty}$] and the apparent volume of distribution (Vd/F) was calculated from the expression Dose/(λz × AUC).

***6β-hydroxycortisol and cortisol*:**

The ratio of endogenous 6β-hydroxycortisol to cortisol in urine is a noninvasive biomarker of CYP3A induction.(Ohno *et al.*, 2000) Since PXR ligands, such as rifampin and hyperforin, induce both CYP2C8 and CYP3A,(Raucy *et al.*, 2002; Park *et al.*, 2004a) the urinary 6β-hydroxycortisol to cortisol ratio serves as a positive control of CYP enzyme induction by St. John's wort.

Statistical analysis:

Rosiglitazone pharmacokinetic parameters were log-transformed where appropriate and rosiglitazone pharmacokinetics in the presence and absence of St. John's wort were compared by paired t-test. Tmax was compared using the Wilcoxon matched pairs test. To examine differences in CYP2C8 induction by genotype, differences in the geometric mean AUC ratio

between wild type (*CYP2C8*1/*1*) and heterozygous (*CYP2C8*1/*2* , *CYP2C8*1/*3*, and *CYP2C8*1/*4*) genotype groups were compared using a one-way ANOVA with Dunnett's multiple comparison test. A two-sided $p \leq 0.05$ was considered significant and all calculations were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Study Subjects:

A total of twenty-seven subjects completed the study (13 male, 14 female); three subjects withdrew during the first rosiglitazone pharmacokinetics study visit due to adverse events. The age, weight, and body mass index of the twenty-seven subjects who completed the study were 24.9 ± 7.8 years (19 – 56), 67.6 ± 12.3 kg (45.5 – 96.2), and 23.3 ± 3.1 kg m^{-2} (18.8 – 29.6), respectively (Mean ± SD (range)). The self reported racial background of the study subjects is as follows: six were African-American, nineteen were Caucasian, and two were Hispanic. Seven of the female subjects were taking oral contraceptives and none of the subjects took complimentary medicine or over the counter medications during the study visits.

Rosiglitazone was tolerated relatively well and the most common adverse event was severe headache. The incidences of severe headache occurred during the control phase of the study in four separate individuals causing three subjects to withdraw from the study. The fourth subject completed the study with no further adverse effects noted. According to subject diaries, all but one dose of St. John's wort (1133/1134 doses, 99.9%) were taken by the 27 individuals. St. John's wort was well tolerated with the most common observed adverse events being dizziness, increased tiredness, and headache. No subjects withdrew from the study due to adverse events associated with St. John's wort.

***Rosiglitazone Pharmacokinetics*:**

Mean log concentration versus time profiles of single dose rosiglitazone (8 mg) in the presence and absence of St. John's wort administration (900 mg daily) are shown in Figure 18. St. John's wort administration significantly affected the disposition of rosiglitazone as the mean AUC and t1/2 decreased by 26% ($p < 0.0001$) and 17 % ($p < 0.0001$), respectively, and CL/F increased by 37% ($p < 0.0001$), while the tmax and Cmax were not different (Figure 19, Table 14). The Vd/F increased ($p = 0.01$) between control and St. John's Wort phases, 14.0 L versus 15.8 L, respectively, but the mean change was only 12%. Subject genotype and sex had no effect on the inducibility of CYP2C8 in the presence of St. John's wort as the magnitude of change in AUC (Figure 20), t1/2, and CL/F did not differ between genotype groups.

6β-hydroxycortisol to cortisol ratio:

The urinary 6β-hydroxycortisol to cortisol ratio significantly increased 1.5 fold, from 7.4 to 11.2 ($p < 0.0001$), in the presence of St. John's wort, providing evidence of CYP induction (See Appendix B, Figure 27). This magnitude of change is similar to previous reports of CYP3A induction due to St. John's wort administration (Roby *et al.*, 2000; Frye *et al.*, 2004). A correlation between CYP2C8 induction, as measured by a change in rosiglitazone AUC, and change in 6β-hydroxycortisol to cortisol ratio was not observed.

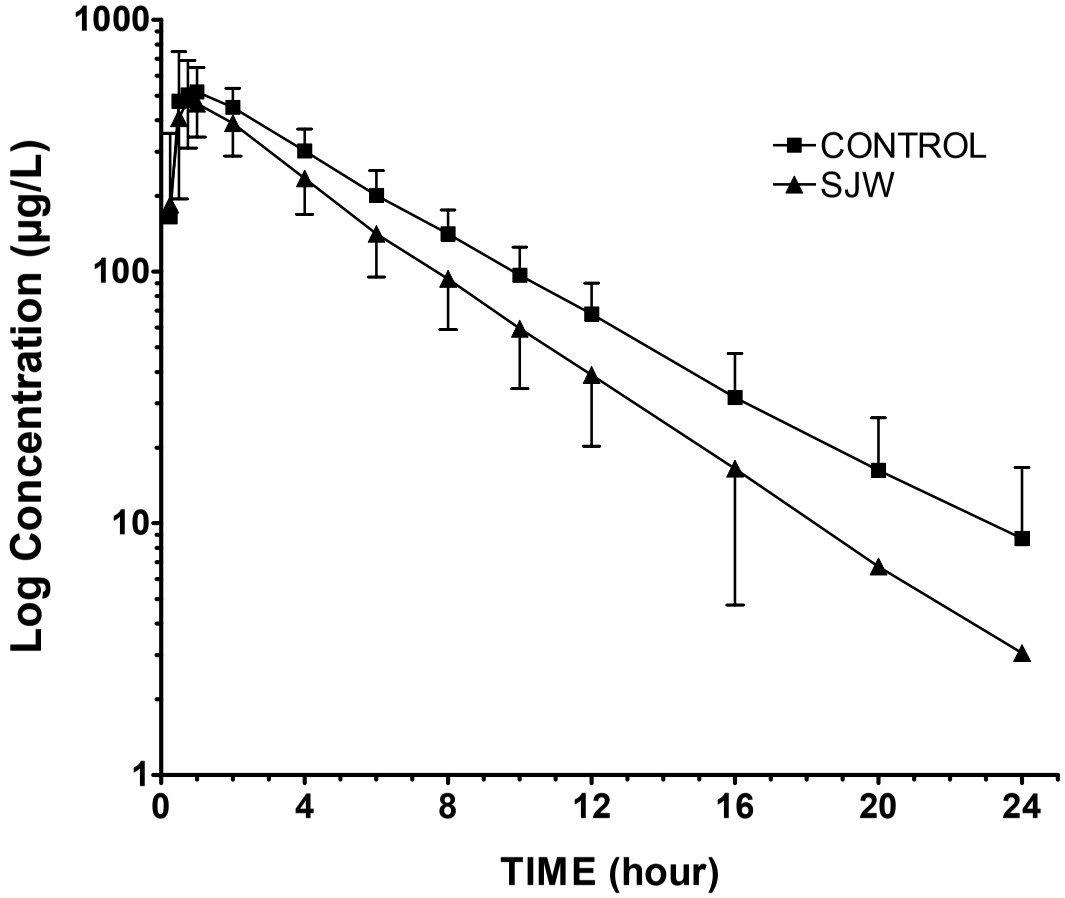


Figure 18 Mean (± S.D.) log concentration versus time profile of rosiglitazone in the presence (*closed triangles*) and absence (*closed squares*) of St. John's wort extract (SJW), 300 mg given three times daily for fourteen days. Data are from 27 healthy subjects.

Table 14 Mean pharmacokinetic parameters of rosiglitazone in the absence and presence of St. John's wort extract, 300 mg three times daily for fourteen days in 27 subjects

Parameter	Control	St. John's Wort	p value	SJW / Control	
				Geometric Mean	90% CI
AUC (hr*μg/l)	3190 ± 641	2375 ± 537	< 0.0001	0.74	0.71 – 0.78
Cl/F (l/hr)	2.6 ± 0.6	3.5 ± 0.7	< 0.0001	1.35	1.28 – 1.42
t1/2 (hr)	3.8 ± 0.9	3.2 ± 0.7	< 0.0001	0.83	0.80 – 0.86
Cmax (μg/l)	613.6 ± 151.0	568.4 ± 139.7	0.18	0.92	0.85 – 1.00
tmax (hr)	0.75 (0.5 – 4)*	0.75 (0.25 – 2)*	0.40	-	-

* tmax given as median and range

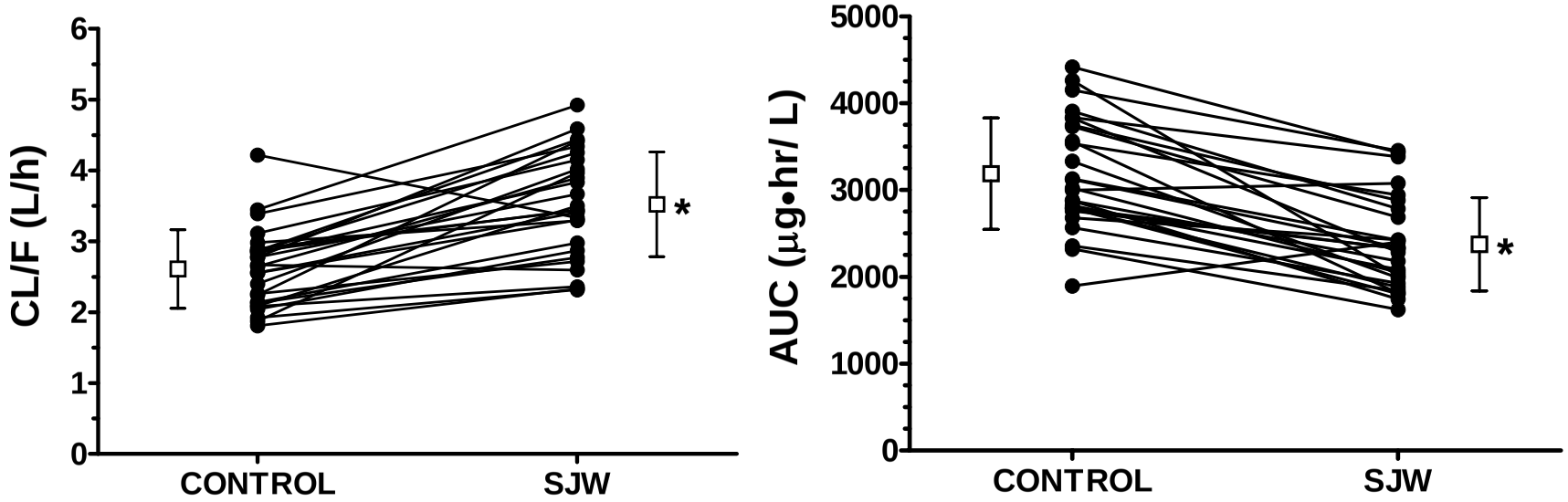


Figure 19 Rosiglitazone (A) Cl/F and (B) AUC in the presence (SJW) and absence (Control) of St. John's wort extract, 300 mg given three times daily for fourteen days in 27 healthy subjects. *p < 0.0001

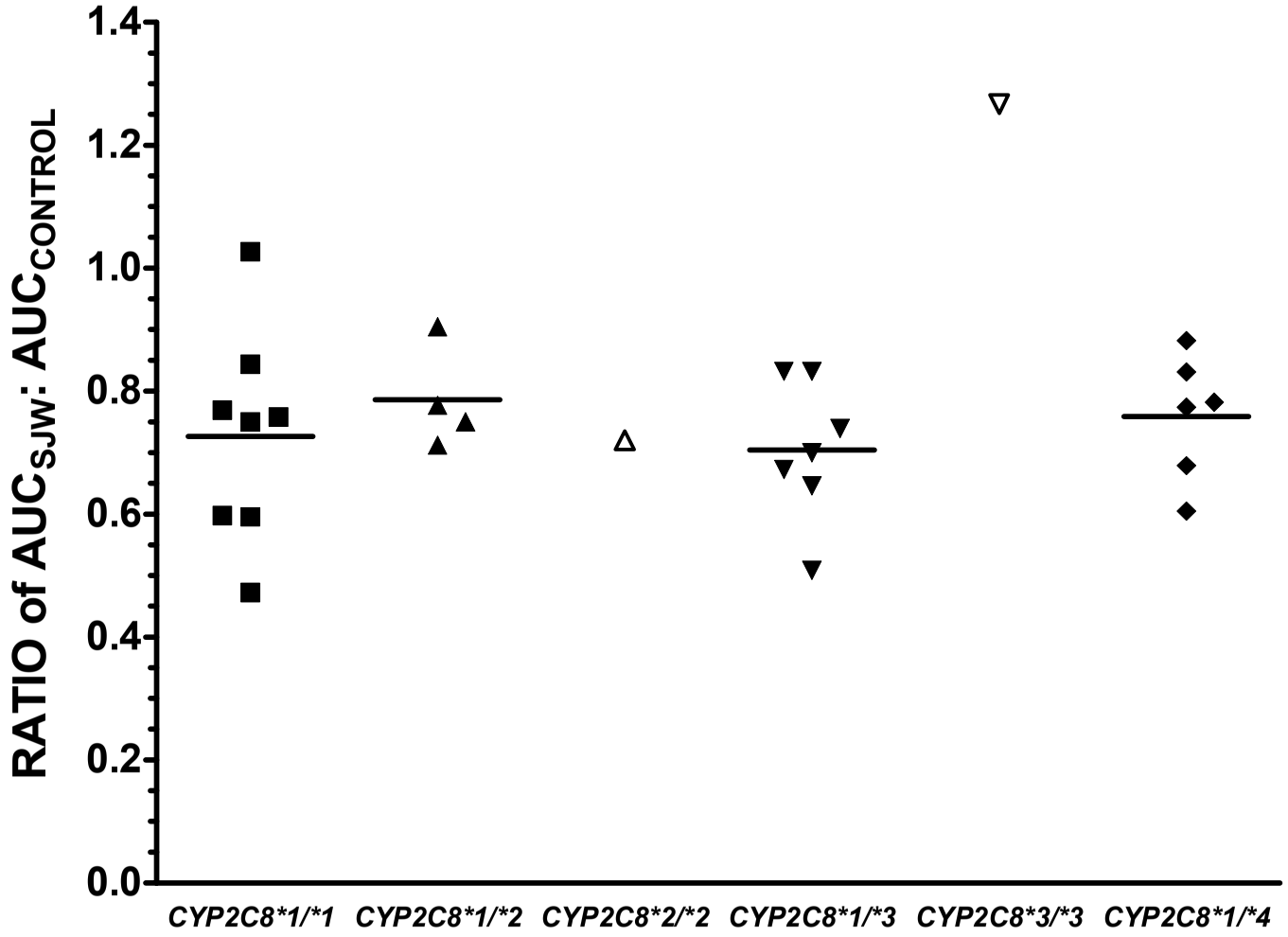


Figure 20 Ratio of rosiglitazone AUC in the presence of St. John's wort (SJW) to rosiglitazone AUC at control visit between the genotypes *CYP2C8*1/*1*, *CYP2C8*1/*2* , *CYP2C8*1/*3*, *CYP2C8*1/*4*, *CYP2C8*3/*3*, *CYP2C8*2/*2.* Horizontal lines indicate median.

Discussion

In the present study, we have shown that St. John's wort increases elimination of the CYP2C8 substrate rosiglitazone. Rosiglitazone exposure was decreased as the plasma AUC and half-life were decreased and the apparent oral clearance was increased in the presence of St. John's wort. St. John's wort administration did not affect rosiglitazone absorption as Cmax and tmax were not different between study visits. Thus, the results of this study demonstrate that St. John's wort induces CYP2C8 activity as determined by rosiglitazone pharmacokinetics.

The results of this study are consistent with previous reports in that St. John's wort administration (900 mg daily for 14 days) has been shown to induce the activities of CYP3A (Durr *et al.*, 2000; Roby *et al.*, 2000; Markowitz *et al.*, 2003), CYP2C9 (Jiang *et al.*, 2004), and CYP2C19 (Wang *et al.*, 2004a; Wang *et al.*, 2004b). Notably, the effect of St. John's wort appears to be greatest for substrates that are metabolized by intestinal CYP enzymes, such as midazolam (Wang *et al.*, 2001; Dresser *et al.*, 2003; Hall *et al.*, 2003) and verapamil (Tannergren *et al.*, 2004). For example, Dresser *et al.*, showed that St. John's wort increased the clearance of midazolam by 2.7 fold after oral administration but only 1.5 fold after intravenous administration (Dresser *et al.*, 2003). St. John's wort appears to have a smaller effect on CYP3A substrates that have low first pass metabolism and high bioavailability, such as imatinib and alprazolam, for which there was an increase in oral clearance of 1.4 and 2.0 fold, respectively (Markowitz *et al.*, 2003; Frye *et al.*, 2004). Thus, the comparatively modest 1.4 fold increase in oral clearance observed with rosiglitazone is probably due to its limited first pass metabolism (Cox *et al.*, 2000) and that CYP2C8 is found primarily in the liver with virtually no expression in the intestine (Lapple *et al.*, 2003).

Previously, Park *et al.*, showed that the PXR ligand rifampin decreased rosiglitazone AUC by greater than 60% and increased its clearance three fold (Park *et al.*, 2004a), which is greater than the 26% decrease in AUC and 35% increase in CL/F reported in the present study. The difference in magnitude of CYP2C8 induction observed with St. John's wort and rifampin is similar to previous reports evaluating CYP3A and CYP2C19 substrates in the presence of these inducers. Rifampin decreases oral midazolam AUC by 96% (Backman *et al.*, 1996), whereas St. John's wort decreases midazolam AUC by only 50% to 65% (Wang *et al.*, 2001; Hall *et al.*, 2003). Similar differences have been shown for imatinib, with an AUC reduction of 54% (Bolton *et al.*, 2004) and 30% (Frye *et al.*, 2004), for rifampin and St. John's wort, respectively. The urinary excretion of 4′-hydroxymephenytoin, the CYP2C19 mediated metabolite of *S*-mephenytoin, was increased by 2 fold in subjects exposed to rifampin (Feng *et al.*, 1998) versus 1.5 fold in subjects administered St. John's wort (Wang *et al.*, 2004b). Our findings are consistent with previous reports that demonstrate St. John's wort is a weaker inducer of CYP enzymes as compared to rifampin.

The ability of St. John's wort to induce CYP2C8 activity is not affected by *CYP2C8* genotype as there were no differences in the magnitude of change in rosiglitazone pharmacokinetics after treatment with St. John's wort. Genotype has been shown to be important for induction of CYP2C19, as the excretion of 4'-hydroxymephenytoin was increased 204% in homozygous wild type subjects but only 70% in heterozygous subjects (Feng *et al.*, 1998). However, our data suggest that St. John's wort induction is not dependent on *CYP2C8* genetic makeup. This may be due to the nature of the polymorphisms in that the *CYP2C8* variant alleles encode amino acid changes while the *CYP2C19* polymorphisms result in no enzyme formation. In contrast, we showed in a previous study that rosiglitazone inhibition by the

selective CYP2C8 inhibitor trimethoprim was modified in subjects possessing the *CYP2C8*3* allele. There was a close relationship between trimethoprim plasma concentration and extent of CYP2C8 inhibition in subjects genotyped as *CYP2C8*1/*1* or **1/*2*, while there was no relationship when subjects expressing the *CYP2C8*3* allele were included (Hruska *et al.*, 2005).

The magnitude of induction by St. John's wort may affect the clinical outcome of rosiglitazone therapy since its blood glucose lowering effect is dose- and concentration-dependent (Balfour and Plosker, 1999). Therefore, patients with type 2 diabetes utilizing rosiglitazone should exercise caution when self administering St. John's wort as it may influence the pharmacodynamic response and clinical outcome of rosiglitazone therapy.

In conclusion, we have demonstrated that CYP2C8 activity, as determined by rosiglitazone pharmacokinetics, is induced by the herbal supplement St. John's wort and that the magnitude of induction is not affected by *CYP2C8* genotype. The use of St. John's wort with CYP2C8 substrates, such as rosiglitazone, should be monitored with caution since concomitant administration may lead to suboptimal therapy.

Acknowledgements:

This project was supported in part by NIH Research Grant R01 MH63458, funded by the National Institute of Mental Health and the Office of Dietary Supplements, and NIH/NCRR/GCRC #5M01RR00082.

Chapter 7

Effect of *CYP2C8* Genotype on Rosiglitazone Metabolism

[Hruska MW, Cheong JA, Amico JA, Langaee TY, and Frye RF. Effect of CYP2C8 genotype on Rosiglitazone Metabolism. *Submitted*.]

Abstract

Objective: The purpose of this study was to evaluate the pharmacokinetics of rosiglitazone in subjects with the *CYP2C8* polymorphisms *CYP2C8*2*, *CYP2C8*3*, and *CYP2C8*4*.

Methods: This single dose pharmacokinetic study involved thirty-eight subjects with the following *CYP2C8* genotypes: *CYP2C8*1/*1* (n = 14), *CYP2C8*1/*2* (n = 5), *CYP2C8*1/*3* (n = 11), *CYP2C8*1/*4* (n = 6), *CYP2C8*2/*2* (n = 1), and *CYP2C8*3/*3* (n = 1). Subjects participated in two previous drug interaction studies (Chapters 5 and 6) in which they were administered a single dose of rosiglitazone 8 mg. Non-compartmental rosiglitazone pharmacokinetic parameter estimates were compared between wild type and heterozygous genotype groups.

Results: CYP2C8 genotype did not affect rosiglitazone exposure as the area under the plasma concentration-time curve (AUC) did not differ between wild type (*CYP2C8*1/*1*), 3164 +/- 625 μg*hr/L (95% Confidence Interval [CI], 2871 – 3457) and heterozygous individuals, *CYP2C8*1/*2*, 3290 +/- 867 μg*hr/L (95% CI, 2464 – 4116), *CYP2C8*1/*3*, 2933 μg*hr/L +/- 519 (95% CI 2654 – 3212) and *CYP2C8*1/*4*, 3230 +/- 658 μg*hr/L (95% CI, 2689 – 3771) (p = 0.71). The individual genotyped as *CYP2C8*3/*3* had the second lowest observed AUC (1896 μg*hr/L); whereas the subject having the *CYP2C8*2/*2* genotype had a higher than average AUC (3733 μg*hr/L), consistent with decreased rosiglitazone clearance. The AUC and formation rate of the metabolites *N*-desmethylrosiglitazone (AUC, p = 0.45; AUC_{NDR}: $AUC_{rosiglitazone}$, p = 0.57) and *para*-O-sulfate-rosiglitazone (p = 0.36, p =0.13) were not different between genotype groups.

Conclusions: The effect of allelic variation on *in vivo* CYP2C8 activity appears to be substrate specific as the pharmacokinetic profile of rosiglitazone is not influenced by heterozygous genotypes. Additional studies are necessary to investigate the influence of homozygous expression of the *CYP2C8*2* and *CYP2C8*3* alleles and the relative contribution of CYP2C8 and CYP2C9 polymorphisms on rosiglitazone metabolism.

Introduction

Cytochrome P450 (CYP) 2C8 is an important enzyme in drug metabolism as it metabolizes the substrates, paclitaxel (Rahman *et al.*, 1994), repaglinide (Bidstrup *et al.*, 2003), amodiaquine (Li *et al.*, 2002), *R*-(-)-ibuprofen (Hamman *et al.*, 1997), rosiglitazone (Baldwin *et al.*, 1999), and amiodarone (Soyama *et al.*, 2002a), and the endogenous compounds retinoic acid (Nadin and Murray, 1999) and arachidonic acid (Dai *et al.*, 2001). This enzyme exhibits polymorphic expression with five allelic variants discovered to date, *CYP2C8*1* (wild type), *CYP2C8*2*, *CYP2C8*3* (Dai *et al.*, 2001), *CYP2C8*4* (Bahadur *et al.*, 2002), and *CYP2C8*5* (Nakajima *et al.*, 2003). The *CYP2C8*2* allele, which is due to Ile269Phe substitution, is primarily found in African Americans at an allele frequency of 0.18, and causes a decreased intrinsic clearance of paclitaxel (Dai *et al.*, 2001). *CYP2C8*3* results from Arg139Lys and Lys399Arg substitutions, is found primarily in Caucasians at an allele frequency from 0.13 to 0.17 (Dai *et al.*, 2001; Martinez *et al.*, 2004), and exhibits decreased *in vitro* CYP2C8 activity (Dai *et al.*, 2001). *CYP2C8*4* is found at a frequency of approximately 0.075 in the Caucasian population, is the consequence of an Ile264Met substitution, and demonstrates a trend towards decreased CYP2C8 activity (Bahadur *et al.*, 2002). The deletion of adenine at nucleotide 475 produces the *CYP2C8*5* allele, which is found in the Japanese population at an allele frequency of 0.0025, and may cause decreased CYP2C8 activity (Nakajima *et al.*, 2003; Ishikawa *et al.*, 2004). Two other variants, *CYP2C8*1A* and *CYP2C8*1B*, are found in the promoter regions and do not affect CYP2C8 activity (Bahadur *et al.*, 2002).

To date, *CYP2C8*3* and *CYP2C8*4* are the only CYP2C8 alleles that have been investigated *in vivo*. Niemi *et al.* demonstrated that individuals with the *CYP2C8*1/*3* genotype had greater clearance of the oral insulin secretagogue repaglinide than those with the

*CYP2C8*1/*1* genotype, while clearance was not different in subjects with the *CYP2C8*1/*4* genotype (Niemi *et al.*, 2003c). Conversely, two studies have shown that carriers of the *CYP2C8*3* allele have decreased clearance of *R*-(-)-ibuprofen. Martínez *et al.*, determined that carriers of the *CYP2C8*3* allele had significantly increased AUC and decreased oral clearance values for *R*-(-)-ibuprofen in a gene-dose effect manner (Martinez *et al.*, 2004). García-Martín *et al.* demonstrated that subjects who possess both the *CYP2C8*3* and *CYP2C9*3* alleles have decreased clearance of *R*-(-)-ibuprofen and racemic ibuprofen as compared to wild type individuals. The investigators determined that polymorphisms of both CYP2C8 and CYP2C9 contribute to variable ibuprofen pharmacokinetics (Garcia-Martin *et al.*, 2004). These studies demonstrate that the influence of polymorphic expression on CYP2C8 activity may be substrate specific, which is similar to what has been shown for CYP2C9 activity (Takanashi *et al.*, 2000). *CYP2C9*3* causes decreased clearance of the CYP2C9 substrates warfarin and flurbiprofen *in vitro* (Yamazaki *et al.*, 1998; Takanashi *et al.*, 2000) and *in vivo* (Takahashi *et al.*, 1998; Lee *et al.*, 2003a), but has no effect on diclofenac clearance (Shimamoto *et al.*, 2000; Takanashi *et al.*, 2000). Since CYP2C8 may exhibit substrate dependent metabolism, it would be important to determine rosiglitazone disposition in the presence of CYP2C8 polymorphisms. Therefore, the purpose of this study is to evaluate the effect of the CYP2C8 polymorphisms, *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4* on rosiglitazone pharmacokinetics.

Methods

Study Subjects

The studies were approved by the Institutional Review Boards at the University of Pittsburgh and University of Florida. Subjects were recruited as previously reported (See Chapters 5 and 6). Briefly, eight subjects were recruited to complete the rosiglitazone with trimethoprim study and one-hundred eleven subjects were screened to select thirty subjects to complete the rosiglitazone and St. John's wort study. Thus, a total of thirty-eight subjects (18 male, 20 female) are available for this analysis. Subjects were determined to be healthy on the basis of past medical history, a physical examination and routine clinical laboratory tests, and were excluded if they had any evidence of abnormal renal or hepatic function, had a BMI > 31 kg m^{-2}, had preexisting medical conditions, or used dietary/herbal supplements or any medications other than oral contraceptives. Women of child bearing age were tested for pregnancy prior to enrollment and on admission for the study visit for exclusionary purposes. Subject age, weight, and body mass index of the thirty-eight subjects who completed the study were 25.6 ± 8.7 years (19 – 57), 68.6 ± 13.2 kg (45.5 – 101.0), and 23.4 ± 3.1 kg m^{-2} (18.8 – 29.6), respectively (Mean ± SD (range)). The self reported racial background of the study subjects are as follows: African-American 7, Asian 1, Caucasian 28, and Hispanic 2. Eight of the female subjects were taking oral contraceptives and none of the subjects took complimentary medicine or over the counter medications during the study visit.

Study Design

Subjects from the previous studies were stratified based on their CYP2C8 genotype into one of 4 groups, *CYP2C8*1*, *CYP2C8*2*, *CYP2C8*3*, or *CYP2C8*4*, containing at least 5 subjects and were administered a single oral dose of rosiglitazone 8 mg. Subjects abstained from alcohol,

caffeine containing foods and beverages, grapefruit or grapefruit juice, and over-the-counter medications for 48 hours prior to the study visit. After an overnight fast, subjects were administered rosiglitazone 8 mg (Avandia®, GlaxoSmithKline, Research Triangle Park, NC, USA) with 8 oz (240 ml) of water at approximately 8:00 AM on study day 1. Subjects fasted for approximately 2 hours after rosiglitazone dosing. Blood samples (10 ml) were obtained before and 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours after drug administration. Blood samples were drawn in EDTA tubes, kept on ice, and centrifuged within two hours of collection at 2500 g, 4^{o}C, for 15 minutes. Plasma was harvested and stored at -70^{o}C until analysis.

Sample Analysis

Rosiglitazone: Rosiglitazone plasma samples were analyzed as described previously (See Chapter 2) (Hruska and Frye, 2004b). Acetonitrile (600 µl) was added to plasma samples (200 µl) spiked with 250 ng of betaxolol (internal standard). Samples were vortex-mixed, centrifuged at 3000 g for 10 minutes, and supernatant was transferred and evaporated to dryness at 45°C under a stream of nitrogen, followed by reconstitution with mobile phase (200 µl). Analytes were monitored with HPLC linked with fluorescence detection under the following conditions: rosiglitazone: excitation wavelength (λ_{ex}) of 247 nm, emission wavelength (λ_{em}) of 367 nm; betaxolol: λ_{ex} of 235 nm, λ_{em} of 310 nm. Separation was achieved with an Alltima Phenyl 5µ 150 mm × 4.6 mm column (Alltech Associates Inc., Deerfield, IL, USA), and an isocratic mobile phase of 10 mM sodium acetate (pH 5): acetonitrile (60:40) delivered at a flow rate of 1 ml/min.

Rosiglitazone metabolites*:* Determination of rosiglitazone metabolites was described previously (Hruska *et al.*, 2005). Briefly, plasma samples (100 µl) were processed with 96-well 0.45µ Captiva® filter plates (Varian Inc, Lake Forest, CA, USA) preloaded with 300 µl of acetonitrile. Internal standard (250 ng) was added to each sample and the plates were vortex-mixed, inverted,

and filtered under vacuum, followed by addition of water (300 µl) to the filtrate. Aliquots (10 µl) were injected onto a Surveyor HPLC system connected to a TSQ Quantum MS/MS system (Thermo, Woburn, MA, USA). Detection was achieved with positive electrospray ionization and data output was captured with Xcalibur® software (Thermo, San Jose, CA, USA). Precursor and product ions $[M + H]^+$ detected were *m/z* 374.2/151 and 344.2/121, for *para*-O-sulfate-rosiglitazone and *N*-desmethylrosiglitazone, respectively. The internal standard was detected with single ion monitoring $[M + H]^+$ at 308.3. Chromatographic separation was performed with a Symmetry C8 5µm, 2.1 × 150 mm column (Waters Corp., Milford, MA, USA) and gradient elution with a mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol, delivered at a flow rate of 200 µl/min. Metabolite concentrations were calculated in arbitrary units (U/l) relative to the internal standard peak area.

Determination of CYP2C8 Genotype: *CYP2C8* genotype was determined as previously described (See Chapter 4) (Hruska *et al.*, 2004). Briefly, DNA was extracted from blood or buccal swab with QIAamp® DNA Mini Kits (Qiagen, Valencia, CA, USA), according to manufacturer protocol. Target DNA was amplified with PCR and products (10 µl) were isolated with streptavidin-coated Sepharose beads. Genotyping analysis and data capture was carried out with a PSQ HS 96 System (Biotage AB, Uppsala, Sweden) and PSQ HS 96 SNP software (Biotage AB, Uppsala, Sweden).

Data Analysis

Rosiglitazone Pharmacokinetics: The rosiglitazone concentration-time data was analyzed using non-compartmental methods. The terminal elimination rate constant (λz) was estimated by nonlinear least squares regression analysis of the terminal portion of the concentration-time data. Apparent elimination half-life was calculated as 0.693/λz. The area under the concentration time

curve (AUC) was calculated by the linear trapezoidal rule with extrapolation to infinity. Apparent oral clearance (CL_O) was determined as [Dose / $AUC_{0-\infty}$] and the apparent oral volume of distribution (Vd/F) was calculated from the expression Dose/($\lambda z \times$ AUC). For both rosiglitazone metabolites, the area under the concentration time curve (AUC) was calculated by the linear trapezoidal rule from 0 to 24 hours. Pharmacokinetic calculations were performed with WinNonlin 2.1 (Pharsight Corp., Mountain View, CA, USA).

Statistical analysis: Rosiglitazone pharmacokinetic parameters were log-transformed where appropriate and wild type (*CYP2C8*1/*1*) subjects were compared to heterozygous carriers of variant alleles using a one-way ANOVA, with Dunnett's multiple comparison test. Tmax was compared using the Kruskal-Wallis test. A p value ≤ 0.05 was considered significant and all calculations were performed using PRISM software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Study Subjects: Allele frequencies of all subjects are reported in Table 15. Adverse event data was reported previously (See Chapters 5 and 6). Briefly, four subjects developed severe headache after ingestion of rosiglitazone, causing three to drop out of the study before blood sampling was completed. Extrapolation of AUC to infinity was less than 5% for these subjects so early withdrawal did not influence characterization of rosiglitazone pharmacokinetics.

Rosiglitazone Pharmacokinetics: The mean log concentration versus time profiles of rosiglitazone, *N*-desmethylrosiglitazone, and *para*-O-sulfate-rosiglitazone in wild type subjects and carriers of *CYP2C8* allelic variants are shown in Figure 21. Rosiglitazone pharmacokinetic data are shown in Table 16. *CYP2C8* genotype did not affect the disposition or absorption of rosiglitazone as the AUC ($p = 0.71$), Cl/F ($p = 0.97$), t1/2 ($p = 0.45$), Cmax ($p = 0.39$), Vd/F ($p =$

0.39), and tmax (p = 0.15) were not different between heterozygous and wild type genotype groups (Table 16, Figure 22). The subjects with the *CYP2C8*2/*2* genotype had a higher than average (*CYP2C8*1/*1*) AUC, while the subject with the *CYP2C8*3/*3* genotype had the second lowest rosiglitazone AUC value observed. The AUC of *N*-desmethylrosiglitazone (p = 0.45) and *para*-O-sulfate-rosiglitazone (p = 0.36) did not differ between heterozygous and wild type genotype groups (Figure 22). The formation rate of metabolites as determined by the ratio of metabolite $AUC_{0\text{-}24}$: rosiglitazone $AUC_{0\text{-}24}$, was not different between wild type and variant *CYP2C8* alleles for *N*-desmethylrosiglitazone (p = 0.57) and *para*-O-sulfate-rosiglitazone (p = 0.13) (See Appendix C, Figure 28).

Table 15 Allele frequencies of *CYP2C8*1*, *CYP2C8*2*, *CYP2C8*3*, and *CYP2C8*4* for subjects (n = 119) recruited at the University of Pittsburgh and the University of Florida.

		Allele Frequency			
Racial Background	**n**	***CYP2C8*1***	***CYP2C8*2***	***CYP2C8*3***	***CYP2C8*4***
African American	14	0.643	0.286	0.036	0.036
Asian	8	1.00	0.00	0.00	0.00
Caucasian	79	0.842	0.00	0.089	0.07
Hispanic	17	0.853	0.00	0.147	0.00
Other	1	1.00	0.00	0.00	0.00
Total	119	0.832	0.034	0.084	0.050

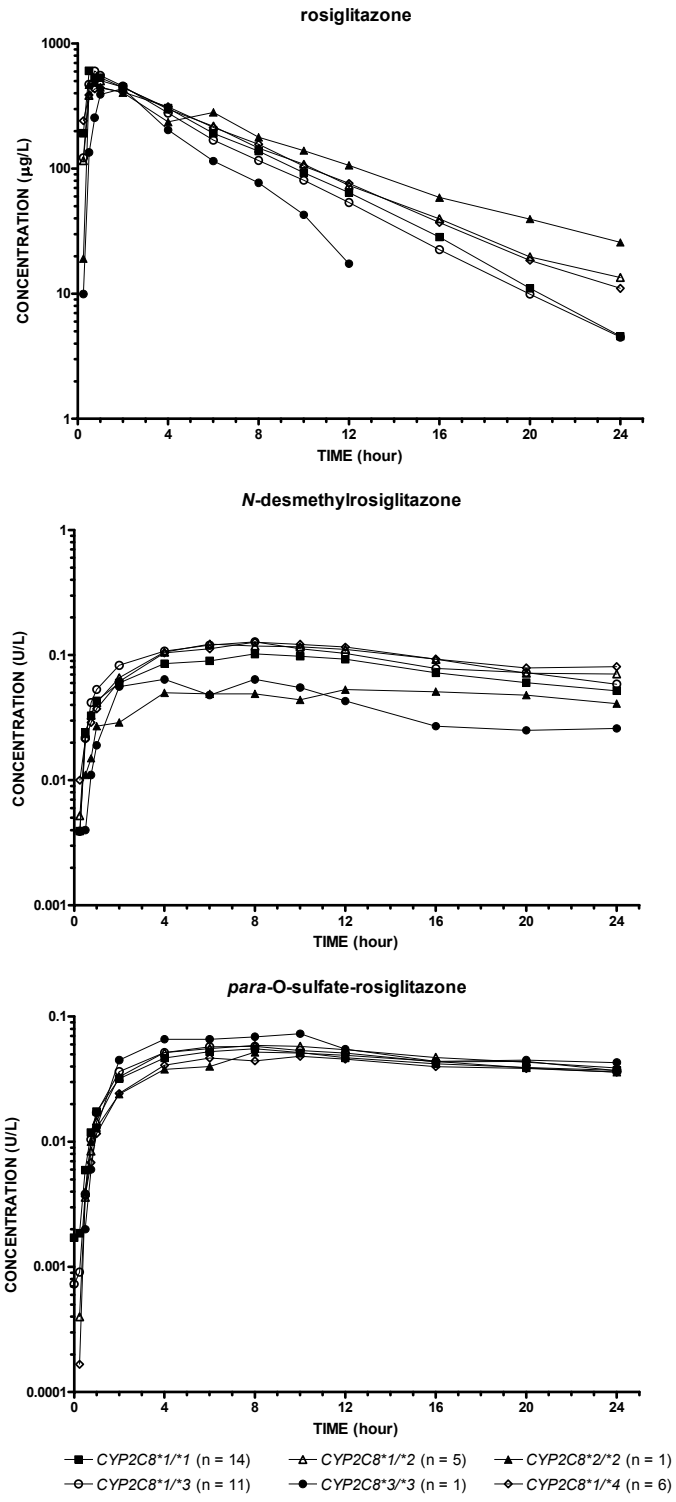


Figure 21 The mean log concentration versus time profile of rosiglitazone, *N*-desmethylrosiglitazone, and *para*-O-sulfate-rosiglitazone in subjects after a single dose of rosiglitazone, 8 mg, with the genotypes: *CYP2C8*1/*1*, n = 14 (closed squares), *CYP2C8*1/*2*, n = 5 (open triangles), *CYP2C8*2/*2*, n = 1 (closed triangles), *CYP2C8*1/*3*, n = 11 (open circles), *CYP2C8*3/*3*, n = 1 (closed circles), and *CYP2C8*1/*4*, n = 6 (open diamonds).

Table 16 Effect of CYP2C8 genotype on rosiglitazone pharmacokinetic parameters. Calculated mean values are reported with 95% confidence intervals (CI) shown in parenthesis, except for subgroups of 1 subject; only individual parameters shown.

Genotype	n	*Rosiglitazone Pharmacokinetic Parameter Estimates*				
		AUC (μg*h/L)	**CL/F (L/h)**	**$t_{1/2}$ (h)**	**Vd/F (L)**	**Cmax (μg/L)**
*CYP2C8*1/*1*	14	3164 (2871 – 3457)	2.63 (2.36 – 2.89)	3.7 (3.3 – 4.0)	13.9 (12.3 – 15.5)	666 (596 – 737)
*CYP2C8*1/*2*	5	3290 (2464 – 4116)	2.57 (1.93 – 3.21)	4.1 (3.7 – 4.5)	15.0 (12.5 – 17.4)	618 (447 – 789)
*CYP2C8*2/*2*	1	3733	2.14	5.6	17.2	491
*CYP2C8*1/*3*	11	2933 (2654 – 3212)	2.81 (2.50 – (3.12)	3.6 (3.2 – 4.1)	14.5 (12.9 – 16.2)	670 (570 – 769)
*CYP2C8*3/*3*	1	1896	4.22	2.0	12.2	436
*CYP2C8*1/*4*	6	3230 (2689 – 3771)	2.56 (2.13 – 3.00)	4.1 (3.6 – 4.6)	14.7 (13.4 – 16.1)	537 (402 – 672)

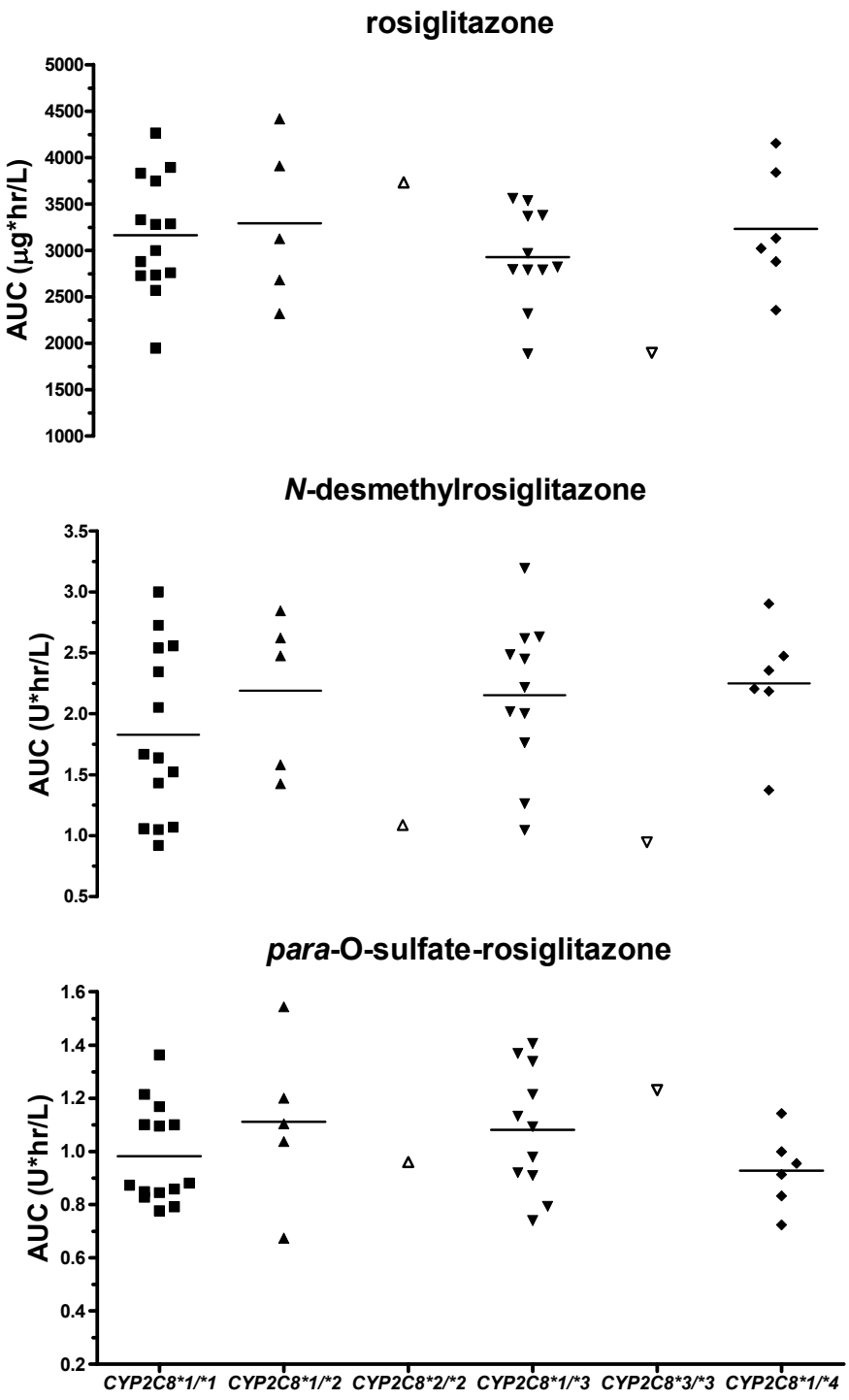


Figure 22 The area under the concentration time curve (AUC) of rosiglitazone, *N*-desmethylrosiglitazone, and *para*-O-sulfate-rosiglitazone in subjects with the genotypes: *CYP2C8*1/*1* (n = 14), *CYP2C8*1/*2* (n = 5), *CYP2C8*2/*2* (n =1), *CYP2C8*1/*3* (n = 11), *CYP2C8*3/*3* (n = 1), and *CYP2C8*1/*4* (n = 6). Horizontal lines indicate mean AUC.

Discussion

The present study is the first to evaluate rosiglitazone metabolism in the presence of *CYP2C8* polymorphisms and to evaluate the effect of the *CYP2C8*2* allele *in vivo*. Rosiglitazone biotransformation is not affected by *CYP2C8* polymorphisms as rosiglitazone pharmacokinetics and metabolite formation after single dose administration does not differ between wild-type individuals and the heterozygote carriers of *CYP2C8* variants. Further investigation is necessary to determine whether homozygous expression of the *CYP2C8*2* and *CYP2C8*3* alleles has any effect on rosiglitazone metabolism.

The results of the present study differ from previous reports, which suggest that the *CYP2C8*3* allele influences the metabolism of repaglinide and *R*-(-)-ibuprofen. Niemi *et al.*, demonstrated that the clearance of repaglinide, a CYP2C8 and CYP3A4 substrate (Bidstrup *et al.*, 2003), is significantly increased in the presence of the *CYP2C8*1/*3* genotype (Niemi *et al.*, 2003c). Conversely, the clearance of *R*-(-)-ibuprofen, which is metabolized by CYP2C8 and CYP2C9 (Hamman *et al.*, 1997), was decreased in a gene-dose manner relative to the *CYP2C8*3* allele. Subjects carrying this allele had significantly lower clearance than wild-type individuals with a more profound effect observed for homozygous carriers of *CYP2C8*3* (Martinez *et al.*, 2004). In the present study, no difference in rosiglitazone AUC was observed for the *CYP2C8*1/*3* genotype, but the *CYP2C8*3/*3* genotype could have a larger impact on rosiglitazone AUC.

The lack of variability in rosiglitazone metabolism in relation to *CYP2C8* genotype suggests that CYP2C8 activity, like CYP2C9 and CYP2D6, may be substrate specific (Takanashi *et al.*, 2000; Wennerholm *et al.*, 2002). The metabolism of the CYP2C9 substrate, flurbiprofen, is significantly decreased *in vitro* (Yamazaki *et al.*, 1998) and *in vivo* (Lee *et al.*, 2003a) in the

presence of the *CYP2C9*3* allele; however, diclofenac, another CYP2C9 substrate, is not affected by this allele *in vitro* (Yamazaki *et al.*, 1998; Takanashi *et al.*, 2000) or *in vivo* (Shimamoto *et al.*, 2000). Wennerholm *et al.* investigated the metabolism of four probes of CYP2D6 activity in the presence of the *CYP2D6*17* allele and found that carriers of this variant allele had altered metabolism for only two of these probes, debrisoquin and dextromethorphan (Wennerholm *et al.*, 2002). CYP2C8 appears to follow a similar pattern of substrate specificity as *in vitro* studies have demonstrated that the metabolism of paclitaxel (Dai *et al.*, 2001; Soyama *et al.*, 2001), but not the *N*-deethylation of amiodarone (Soyama *et al.*, 2002a) is decreased in the presence of the *CYP2C8*3* allele. *In vivo*, the *CYP2C8*3* allele appears to increase repaglinide clearance (Niemi *et al.*, 2003c), but in the present study this allele does not influence rosiglitazone clearance.

Although CYP2C8 variants do not appear to effect rosiglitazone pharmacokinetics, the presence of CYP2C9 variants may have an effect since this enzyme contributes to a minor extent to rosiglitazone metabolism (Baldwin *et al.*, 1999). Metabolism of *R*-(-)-ibuprofen, which is unidirectionally converted up to 63% *in vivo* (Lee *et al.*, 1985) to the CYP2C9 substrate, *S*-(+)-ibuprofen (Hamman *et al.*, 1997), demonstrates the impact of CYP2C9 variants. García-Martín *et al.*, determined the metabolism of racemic ibuprofen, *R*-(-)-ibuprofen, and *S*-(+)-ibuprofen were dependent on both CYP2C8 and CYP2C9 polymorphisms, as the *CYP2C8*3* and *CYP2C9*3* alleles both contributed to the reduced clearance of these substrates (Garcia-Martin *et al.*, 2004). Individuals with the *CYP2C8*1/*1* genotype and at least one copy of the *CYP2C9*3* allele exhibited a reduction of racemic and *R*-(-)-ibuprofen clearance; whereas *CYP2C8*3* carriers, who were *CYP2C9*1/*1*, exhibited no changes in *R*-(-)-ibuprofen clearance (Garcia-Martin *et al.*, 2004). Further, in the study by Martinez *et al.*, all of the subjects presenting with

the *CYP2C8*3* allele had at least one copy of the *CYP2C9*2* allele, but none of the CYP2C8 wild type individuals carried a CYP2C9 mutation (Martinez *et al.*, 2004). These results suggest the pharmacokinetics of substrates metabolized by both CYP2C8 and CYP2C9, such as rosiglitazone and ibuprofen, are impacted by the polymorphic expression of both enzymes. Further, linkage of the *CYP2C8*3* and *CYP2C9*2* alleles, which occurs in approximately 96% of *CYP2C8*3* carriers (Yasar *et al.*, 2002), may also influence rosiglitazone pharmacokinetics. Further evaluation of rosiglitazone metabolism by CYP2C8 and CYP2C9 variants is warranted to determine the genetic contribution of each of these enzymes.

Rosiglitazone metabolism by individuals with *CYP2C8*1/*2* genotype is not consistent with the *in vitro* literature, which demonstrated *CYP2C8*2* has a reduced intrinsic clearance of paclitaxel (Dai *et al.*, 2001). Like the *CYP2C8*3/*3* genotype, the *CYP2C8*2/*2* genotype may have a greater effect on rosiglitazone AUC than observed in heterozygous individuals, but further study is required. If the *CYP2C8*2/*2* genotype does present with decreased activity, it may cause unwanted adverse events, such as edema during rosiglitazone therapy (Idris *et al.*, 2003), or exaggerated pharmacodynamic effects of other CYP2C8 substrates. Additional studies evaluating the *in vivo* activity of *CYP2C8*2* variants, especially homozygous carriers is necessary.

Finally, the present study supports previous *in vitro* (Bahadur *et al.*, 2002) and *in vivo* (Niemi *et al.*, 2003c) data demonstrating *CYP2C8*4* does not influence CYP2C8 activity as the mean rosiglitazone AUC in this sample population was approximately the same as the wild type individuals. It appears that this variant allele, although present at an allele frequency of approximately 7.5% (Bahadur *et al.*, 2002), does not influence CYP2C8 mediated metabolism.

In conclusion, this study demonstrates the influence of polymorphic expression on CYP2C8 activity may be substrate specific as rosiglitazone pharmacokinetics, unlike other CYP2C8 substrates, are not different between heterozygous and wild type individuals. Additional studies investigating the influence of homozygous expression of the *CYP2C8*2* and *CYP2C8*3* alleles and the relative contribution of CYP2C8 and CYP2C9 polymorphisms would be necessary to fully understand their role in rosiglitazone metabolism.

Chapter 8

Conclusions and Future Directions

The purpose of this research was to investigate the utility of rosiglitazone as an *in vivo* probe of CYP2C8 activity. To accomplish this goal, novel methods were developed to determine rosiglitazone and trimethoprim plasma concentrations and to identify *CYP2C8* polymorphisms. The disposition of rosiglitazone was utilized as a marker of CYP2C8 activity in the presence of the CYP2C8 inhibitor, trimethoprim, and the CYP inducer, St. John's wort extract, and the impact of *CYP2C8* genotype on these interactions was evaluated. Finally, basal rosiglitazone pharmacokinetic parameters were compared between genotype groups to determine the influence of *CYP2C8* polymorphisms on *in vivo* rosiglitazone metabolism.

To measure CYP2C8 activity through the evaluation of rosiglitazone metabolism, a method utilizing fluorescence detection was developed to detect rosiglitazone plasma concentrations. Advantages of this method relative to published methods include the use of protein precipitation, which simplifies sample processing, the use of small volumes of plasma, and the application of a commercially available internal standard. To determine trimethoprim plasma concentrations and their correlation with CYP2C8 inhibition, an assay employing ultraviolet detection was developed. Advantages include the use of simple protein precipitation, which does not require further preparation, consistent recovery, and direct applicability to the determination of trimethoprim plasma concentrations in pharmacokinetic studies or after administration of high dose therapy. To ascertain the influence of *CYP2C8* genotype on rosiglitazone metabolism, a genotyping method was developed as a screening tool for *CYP2C8* allelic variants. This method utilizes Pyrosequencing technology, which offers the primary advantage over other genotyping methods of determining the direct sequence of target DNA. Pyrosequencing is a cost effective and less time consuming genotyping method that allows for high throughput analysis and precise determination of *CYP2C8* variants.

The first interaction study, which evaluated the *in vitro* and *in vivo* effects of trimethoprim on rosiglitazone metabolism, determined that trimethoprim is an effective CYP2C8 inhibitor *in vitro* and significantly inhibits rosiglitazone metabolism *in vivo*. Trimethoprim, a competitive inhibitor, is more potent *in vitro* compared to the CYP2C8/9/19 inhibitor, gemfibrozil, which inhibits CYP2C8 by a noncompetitive mechanism; however, *in vivo* trimethoprim has a less profound effect on rosiglitazone metabolism. The difference in extent of inhibition observed between trimethoprim and gemfibrozil is most likely related to the inhibitor steady state concentration, which for gemfibrozil exceeds the inhibition constant (Ki) but for trimethoprim does not. Finally, carriers of the *CYP2C8*3* allele did not exhibit inhibition by trimethoprim in a concentration dependent manner, whereas non-carriers of this did.

The second study evaluated the effect of St. John's wort extract administration on rosiglitazone metabolism in healthy subjects genotyped for *CYP2C8* polymorphisms. St. John's wort administration significantly decreases rosiglitazone exposure, but the magnitude of induction ($AUC_{control}/AUC_{St.\ John's\ wort}$) was not dependent on *CYP2C8* genotype. This study illustrates St. John's wort extract induces rosiglitazone metabolism and may influence the pharmacodynamic response of rosiglitazone.

Finally, analysis of basal rosiglitazone pharmacokinetic parameters for all subjects was conducted to determine the effect of genotype on rosiglitazone metabolism. Exposure to rosiglitazone was not different between wild type and heterozygous carriers of *CYP2C8* variant alleles, which differs from previous reports of substrates in the presence *CYP2C8* variants. Interestingly, homozygous carriers of the *CYP2C8*2* and *CYP2C8*3* alleles appear to have a more profound affect on rosiglitazone metabolism, but further investigation is necessary. Therefore, the effect of *CYP2C8* genotype on *in vivo* activity may be substrate specific, resulting

in no effect on rosiglitazone metabolism, or the effect of genotype on rosiglitazone metabolism may only be relevant in carriers of two allelic variants.

Rosiglitazone is a suitable probe to evaluate drug interactions involving CYP2C8, as the pharmacokinetic parameters of rosiglitazone are affected by the CYP2C8 modulators trimethoprim, gemfibrozil, rifampin, and St. John's wort extract. Rosiglitazone is a more suitable probe of hepatic CYP2C8 activity versus other potential *in vivo* CYP2C8 probes, paclitaxel and repaglinide. Paclitaxel, an antineoplastic agent, cannot be safely administered to healthy volunteers and CYP3A contributes to the metabolism of repaglinide, which may complicate the interpretation of CYP2C8 induction studies, since both enzymes are induced via the orphan nuclear receptor PXR. Rosiglitazone offers advantages over these substrates as it has a favorable pharmacokinetic profile and has close to complete bioavailability allowing its use as an *in vivo* probe of CYP2C8 activity in drug-drug and drug-herb interaction studies.

The major polymorphisms of *CYP2C8*, unlike *CYP2C19* and *CYP2D6*, do not abolish enzyme production. Since there are no major changes in CYP2C8 production, there are no apparent PM and EM phenotypes. However, like CYP2C9 and *CYP2D6*17*, changes in CYP2C8 activity in the presence of allelic variants appear to be substrate specific as only some substrates are affected by polymorphic expression. Since the major *CYP2C8* allelic variants do not abolish enzyme production, changes in the amino acid structure may affect the enzyme active site and influence metabolism in a substrate-dependent manner. These alterations of the CYP2C8 active site may help explain why paclitaxel metabolism *in vitro* and repaglinide metabolism *in vivo* are affected by polymorphic expression, but amiodarone metabolism *in vitro* and rosiglitazone metabolism *in vivo* are not. The location of these amino acid substitutions in the CYP2C8 protein may also help explain why the inhibitory effects of trimethoprim, but not

the inductive effects of St. John's wort extract, appear to be affected by *CYP2C8* genotype. Since *CYP2C8* variants do not eliminate enzyme production, induction is not affected by *CYP2C8* genotype, but changes of the enzyme active site may affect the efficacy of a competitive inhibitor, such as trimethoprim. Therefore, rosiglitazone may not be a suitable probe to investigate *CYP2C8* polymorphisms as no changes in the pharmacokinetic profile of rosiglitazone in the presence of these mutations are observed.

Future research is required to confirm the findings of this work and better characterize *CYP2C8* polymorphisms *in vitro* and *in vivo*. *In vitro*, the role of CYP2C8 variants on the metabolism of the substrates *R*-(-)-ibuprofen, rosiglitazone, and repaglinide should be investigated. By evaluating these substrates in the presence of *CYP2C8* polymorphisms, the possibility of substrate dependent CYP2C8 metabolism may be explored. Further, the orientation of each substrate in the CYP2C8 enzyme active site should be investigated to better understand the probable cause of substrate specific effects. *In vivo*, rosiglitazone metabolism in the presence of *CYP2C8* homozygous variant carriers should be assessed to determine if a relationship exists. The extent of rosiglitazone metabolism by CYP2C9 should be evaluated to determine the relative contributions of CYP2C8 and CYP2C9, which would further support the use of rosiglitazone as an *in vivo* CYP2C8 probe. Finally, the individual and combined effects of *CYP2C8* and *CYP2C9* polymorphisms should be evaluated to determine their role in rosiglitazone metabolism.

In summary, the results of the present work indicate that rosiglitazone is suitable for use as an *in vivo* probe of CYP2C8 activity in the presence of potential CYP2C8 inducers and inhibitors; however, it may not be a suitable probe of CYP2C8 activity in the presence of the known *CYP2C8* polymorphisms. The clinical effect of CYP2C8 substrates may be influenced by inhibitors and inducers and the use of rosiglitazone as a CYP2C8 probe may lead to the identification of these potential drug interactions.

APPENDIX A

Individual Subject Data and Additional Figures from the Rosiglitazone-Trimethoprim Drug Interaction Study

Table 17 Individual rosiglitazone pharmacokinetic parameters in the presence and absence of trimethoprim 200 mg twice daily for five days in 8 subjects

Rosiglitazone Pharmacokinetic Parameters

SN	Control	Trimethoprim	SN	Control	Trimethoprim
AUC (μg*h/L)			**Cmax (μg/L)**		
1	3377	3883	1	1104.2	603.1
2	3286	4616	2	766.9	688.2
3	1948	2490	3	660.5	513.1
4	2318	2547	4	388.3	500.5
5	2735	5117	5	381.2	585.6
6	3281	4624	6	697.3	635.3
7	3368	3118	7	809.6	584.3
8	1886	2753	8	586.3	625.4
Mean	**2774**	**3643**	**Mean**	**674.3**	**591.9**
S.D.	**645**	**1051**	**S.D.**	**235.4**	**62.2**
$t_{1/2}$ (h)			**tmax (h)**		
1	2.6	3.9	1	0.75	2.00
2	3.4	4.2	2	0.50	1.00
3	2.7	3.2	3	0.50	0.75
4	3.4	4.2	4	1.00	0.50
5	3.6	5.4	5	4.00	2.00
6	4.0	5.4	6	0.50	0.75
7	3.7	3.3	7	0.75	2.00
8	3.0	4.0	8	0.75	0.75
Mean	**3.3**	**4.2**	**Median**	**0.75**	**0.88**
S.D.	**0.5**	**0.8**	**Range**	**(0.5 – 4.0)**	**(0.5 – 2.0)**
Vd (L)					
1	9.5	12.0			
2	12.5	10.8			
3	16.5	15.4			
4	18.4	19.4			
5	15.7	12.2			
6	14.4	13.7			
7	13.3	12.4			
8	18.9	16.8			
Mean	**14.9**	**14.1**			
S.D.	**3.1**	**2.9**			

Table 18 Individual rosiglitazone metabolite pharmacokinetic parameters in the presence and absence of trimethoprim 200 mg twice daily for five days in 8 subjects

Rosiglitazone Metabolite Pharmacokinetic Parameters					
SN	**Control**	**Trimethoprim**	**SN**	**Control**	**Trimethoprim**
***Para*-O-sulfate-rosiglitazone (POS)**					
AUC (U*h/L)			**AUC Ratio (POS:Rosiglitazone)**		
1	16.9	17.6	1	5.0	4.5
2	19.1	15.5	2	5.9	3.4
3	10.8	12.5	3	5.5	5.0
4	17.9	19.4	4	7.8	7.6
5	14.0	12.8	5	5.2	2.6
6	13.9	10.0	6	4.3	2.2
7	15.0	15.3	7	4.5	4.9
8	11.1	10.6	8	5.8	3.9
Mean	**14.9**	**14.2**	**Mean**	**5.5**	**4.3**
S.D.	**3.0**	**3.3**	**S.D.**	**1.1**	**1.7**
***N*-desmethylrosiglitazone (NDR)**					
AUC (U*h/L)			**AUC Ratio (NDR:Rosiglitazone)**		
1	14.3	18.2	1	4.2	4.7
2	17.8	18.2	2	5.5	4.0
3	12.8	7.2	3	6.5	2.9
4	15.8	11.0	4	6.9	4.3
5	16.9	18.1	5	6.3	3.7
6	18.2	9.3	6	5.7	2.1
7	11.7	8.0	7	3.5	2.6
8	17.3	15.1	8	9.1	5.5
Mean	**15.6**	**13.1**	**Mean**	**6.0**	**3.7**
S.D.	**2.4**	**4.8**	**S.D.**	**1.7**	**1.2**

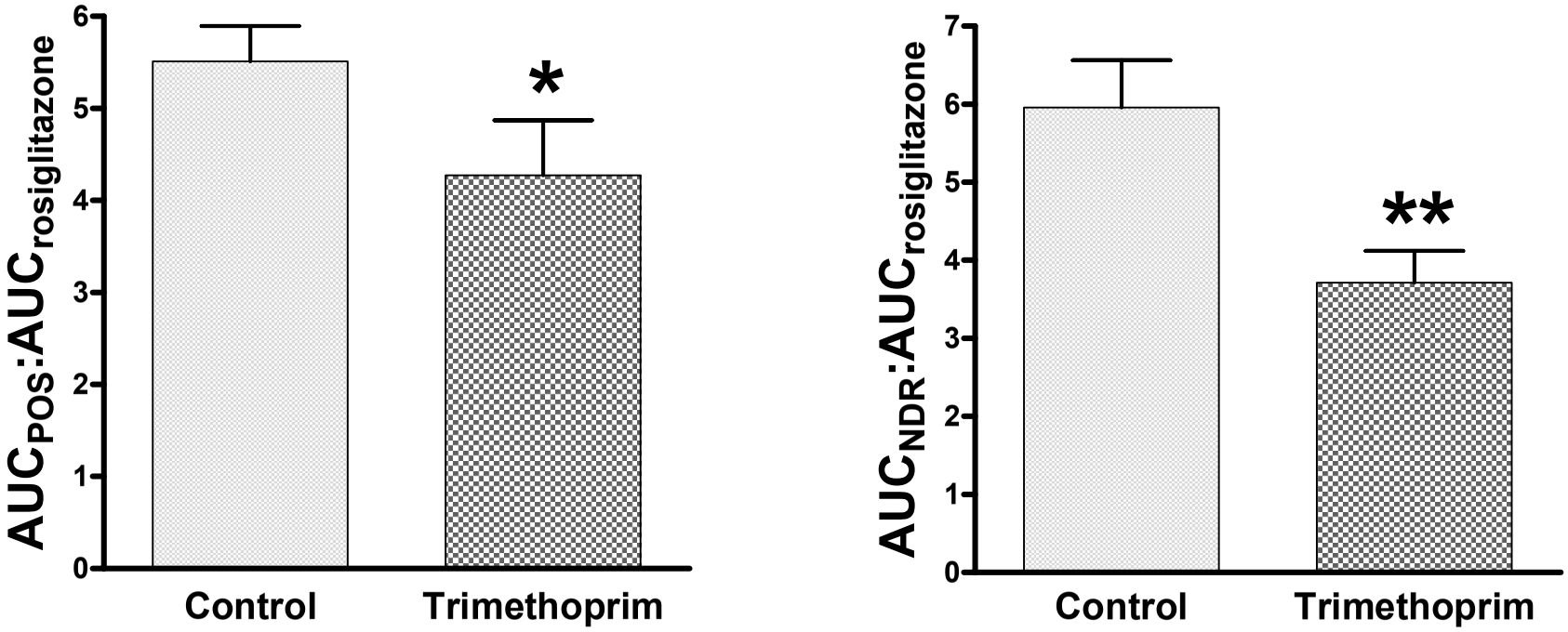


Figure 23 Formation rate of rosiglitazone metabolites after rosiglitazone 8 mg in the absence (Control) and presence (Trimethoprim) of trimethoprim 200 mg twice daily for 5 days in eight subjects. POS: *para*-O-sulfate rosiglitazone NDR: *N*-desmethylrosiglitazone; * p = 0.02; ** p = 0.004

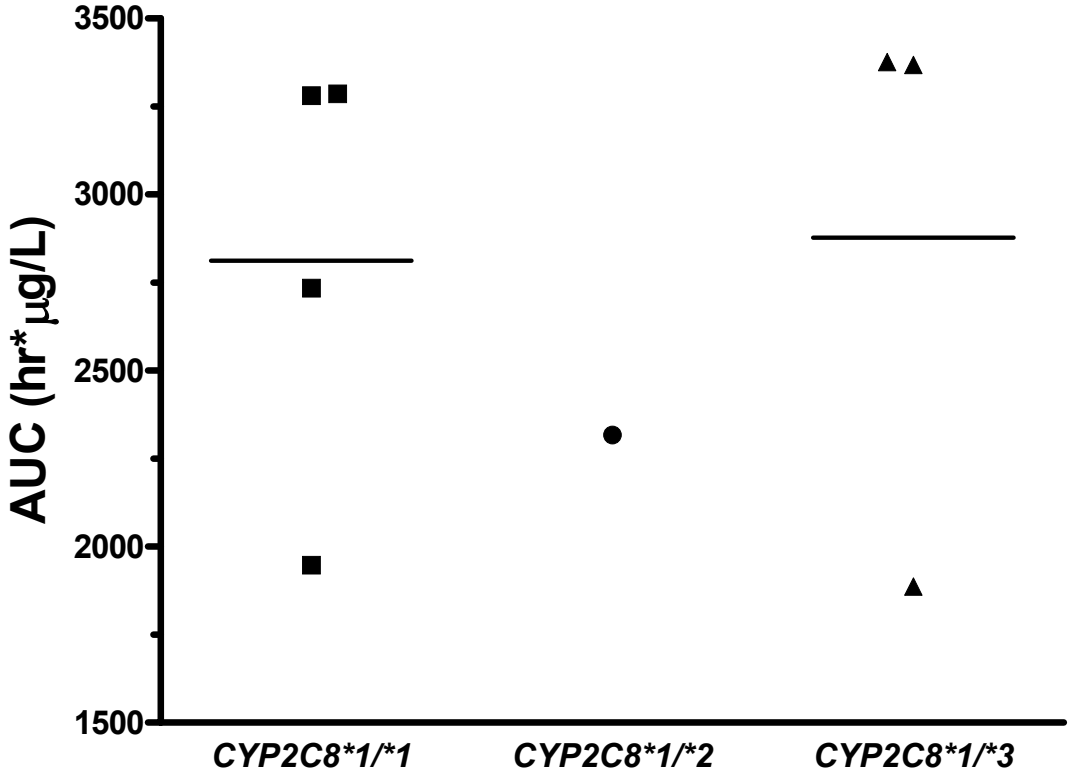


Figure 24 Rosiglitazone AUC in subjects with *CYP2C8*1/*1*, *CYP2C8*1/*2*, and *CYP2C8*1/*3* genotypes. Line indicates mean.

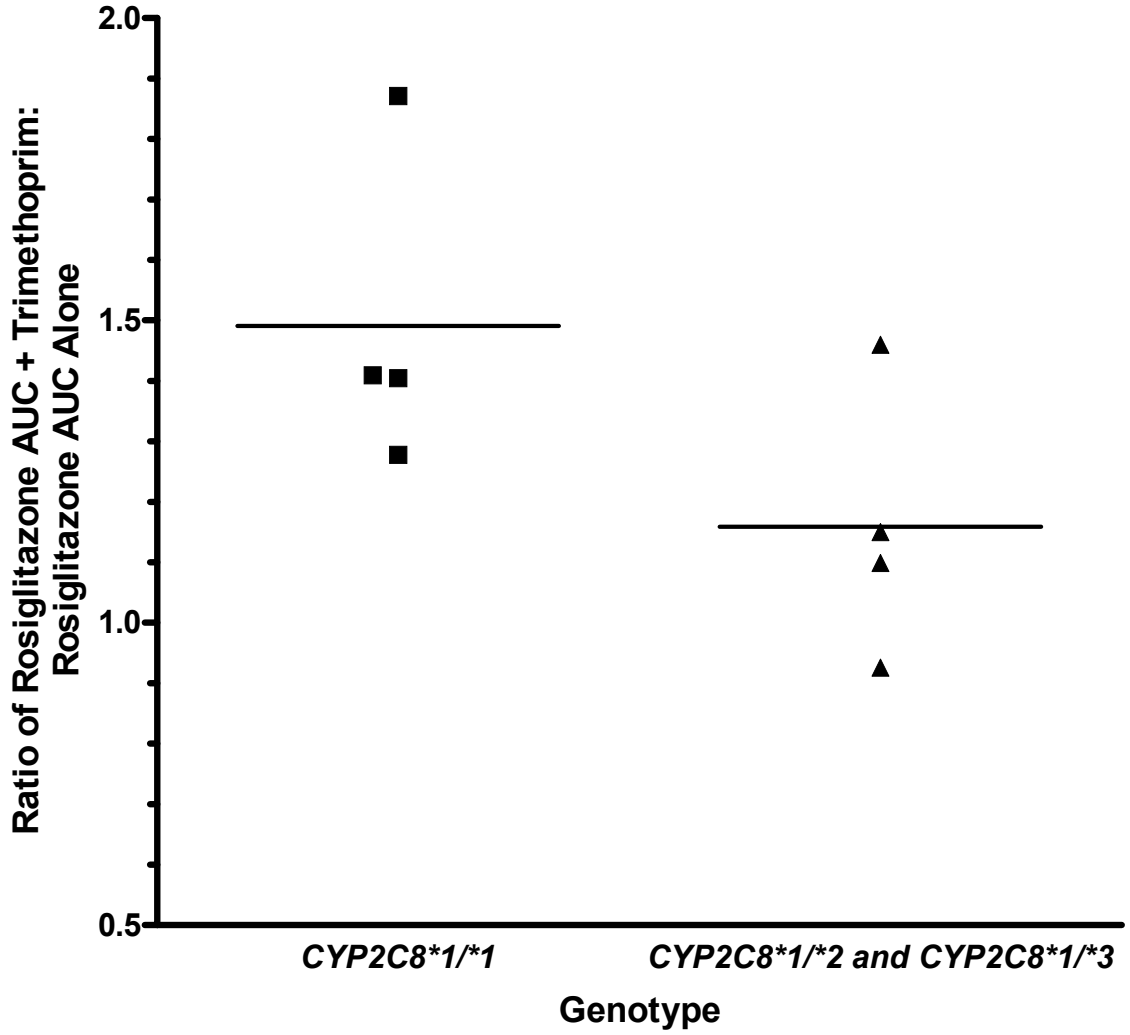


Figure 25 Fold increase of rosiglitazone AUC in the presence trimethoprim in subjects with *CYP2C8*1/*1* and *CYP2C8*1/*2* or *CYP2C8*1/*3* genotypes. Horizontal lines indicate mean.

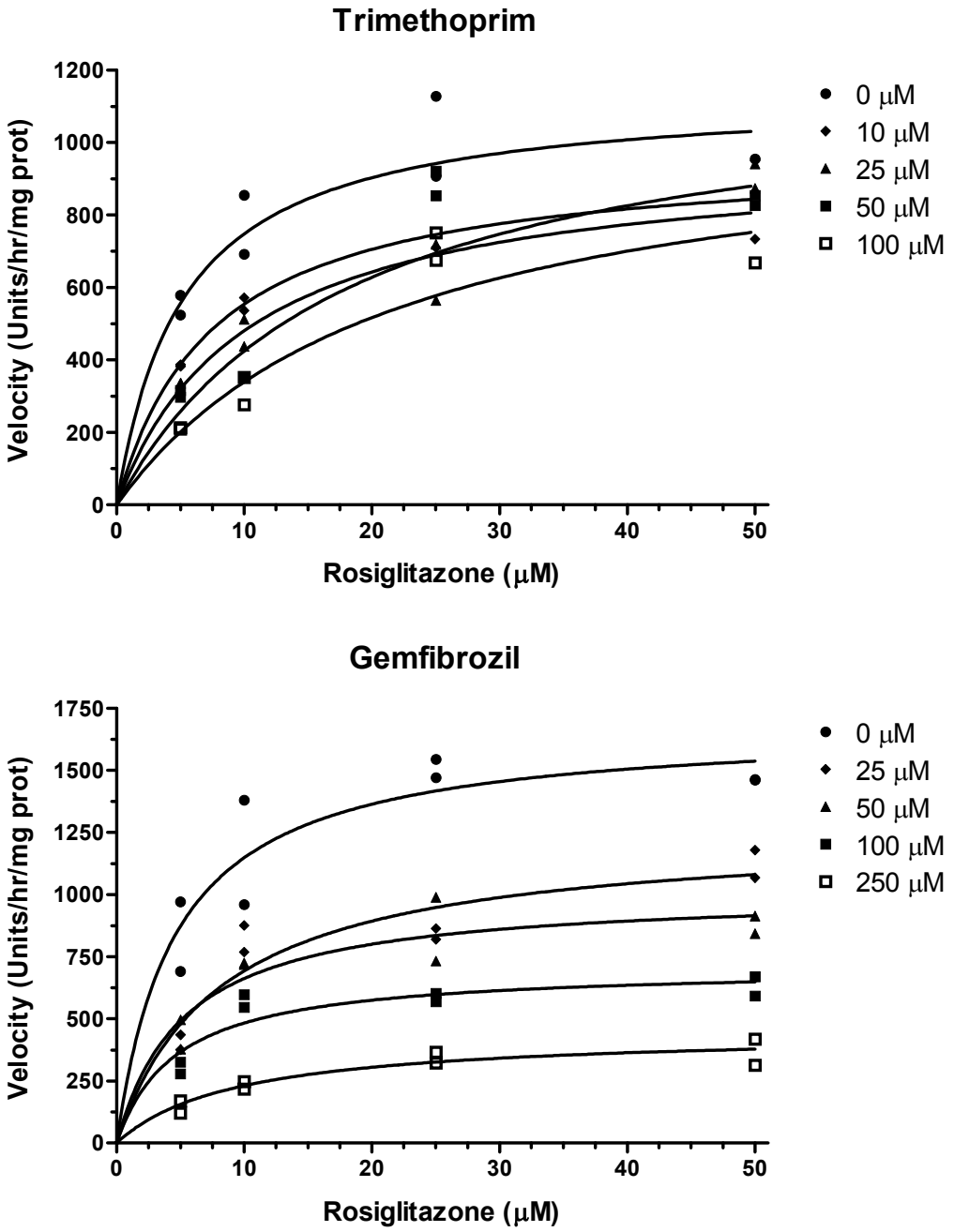


Figure 26 Michaelis-Menten plots of rosiglitazone in the presence of trimethoprim and gemfibrozil in human liver microsomes.

APPENDIX B

Individual Subject Data and Additional Figures from Rosiglitazone-St. John's wort Interaction Study

Table 19 Individual AUC (μg*h/L) and Cl/F (L/h) of rosiglitazone 8 mg in the presence and absence of St. John's wort extract, 300 mg three times daily for fourteen days in 27 subjects

SN	Control	St. John's wort	SN	Control	St. John's wort
AUC (μg*h/L)			**Cl/F (L/h)**		
573-003	3749	2883	573-003	2.1	2.8
573-004	2759	2328	573-004	2.9	3.4
573-005	3832	2284	573-005	2.1	3.5
573-006	2879	2181	573-006	2.8	3.7
573-007	1896	2402	573-007	4.2	3.3
573-011	2789	1803	573-011	2.9	4.4
573-013	2569	1927	573-013	3.1	4.2
573-017	3733	2687	573-017	2.1	3.0
573-025	3125	2344	573-025	2.6	3.4
573-029	3331	1991	573-029	2.4	4.0
573-034	4266	2016	573-034	1.9	4.0
573-035	2998	3079	573-035	2.7	2.6
573-036	3561	1812	573-036	2.2	4.4
573-037	3020	2051	573-037	2.6	3.9
573-038	2794	1881	573-038	2.9	4.3
573-042	4417	3432	573-042	1.8	2.3
573-046	3130	2423	573-046	2.6	3.3
573-055	2358	1843	573-055	3.4	4.3
573-062	3910	2786	573-062	2.0	2.9
573-065	3535	2942	573-065	2.3	2.7
573-069	2682	2427	573-069	3.0	3.3
573-072	3838	3385	573-072	2.1	2.4
573-074	4155	3453	573-074	1.9	2.3
573-077	2879	1742	573-077	2.8	4.6
573-091	2791	2323	573-091	2.9	3.4
573-094	2320	1624	573-094	3.4	4.9
573-099	2821	2085	573-099	2.8	3.8
Mean	**3190**	**2375**	**Mean**	**2.6**	**3.5**
S.D.	**641**	**537**	**S.D.**	**0.6**	**0.7**

Table 20 Individual $t_{1/2}$ (h) and Vd (L) of rosiglitazone 8 mg in the presence and absence of St. John's wort extract, 300 mg three times daily for fourteen days in 27 subjects

SN	Control	St. John's wort	SN	Control	St. John's wort
$t_{1/2}$ (h)			**Vd (L)**		
573-003	3.3	2.7	573-003	10.2	10.6
573-004	4.2	3.0	573-004	17.7	14.7
573-005	2.8	2.4	573-005	8.6	12.1
573-006	3.3	2.6	573-006	13.2	13.7
573-007	2.0	2.2	573-007	12.2	10.6
573-011	4.2	4.9	573-011	17.5	31.5
573-013	3.2	2.8	573-013	14.2	17.0
573-017	5.6	4.5	573-017	17.2	19.2
573-025	4.2	3.5	573-025	15.7	17.5
573-029	3.2	2.8	573-029	11.1	16.4
573-034	3.3	2.5	573-034	9.0	14.5
573-035	4.8	4.0	573-035	18.4	15.1
573-036	5.4	3.2	573-036	17.5	20.3
573-037	4.4	3.6	573-037	16.8	20.5
573-038	3.1	2.5	573-038	12.9	15.5
573-042	4.8	4.5	573-042	12.5	15.1
573-046	4.2	3.2	573-046	15.6	15.4
573-055	3.2	2.5	573-055	15.7	15.8
573-062	4.1	3.3	573-062	12.1	13.6
573-065	3.4	2.7	573-065	11.0	10.4
573-069	3.8	3.4	573-069	16.3	16.4
573-072	4.7	4.2	573-072	14.3	14.3
573-074	4.4	3.6	573-074	12.2	11.9
573-077	3.4	2.6	573-077	13.8	17.5
573-091	4.0	3.0	573-091	16.6	14.9
573-094	2.5	2.6	573-094	12.7	18.4
573-099	3.3	2.5	573-099	13.4	13.7
Mean	**3.8**	**3.2**	**Mean**	**14.0**	**15.8**
S.D.	**0.9**	**0.7**	**S.D.**	**2.8**	**4.1**

Table 21 Individual Cmax (μg/L) and tmax (h) of rosiglitazone 8 mg in the presence and absence of St. John's wort extract, 300 mg three times daily for fourteen days in 27 subjects

SN	Control	St. John's wort	SN	Control	St. John's wort
Cmax (μg/L)			**tmax (h)**		
573-003	703.2	644.2	573-003	0.75	2.00
573-004	501.3	550.1	573-004	0.75	0.50
573-005	884.5	791.2	573-005	0.50	0.25
573-006	683.0	619.2	573-006	0.50	1.00
573-007	436.1	615.4	573-007	2.00	0.50
573-011	816.2	240.6	573-011	0.50	0.50
573-013	476.1	581.4	573-013	2.00	0.50
573-017	491.2	521.8	573-017	0.75	0.50
573-025	489.9	570.6	573-025	2.00	0.75
573-029	694.0	726.1	573-029	0.50	0.50
573-034	910.4	545.4	573-034	0.50	0.50
573-035	651.3	613.3	573-035	0.75	0.75
573-036	546.4	326.8	573-036	0.75	2.00
573-037	471.9	410.2	573-037	1.00	1.00
573-038	573.9	475.2	573-038	0.75	1.00
573-042	775.0	532.6	573-042	1.00	2.00
573-046	641.6	551.8	573-046	0.50	1.00
573-055	302.2	419.8	573-055	4.00	2.00
573-062	805.7	761.5	573-062	0.50	0.50
573-065	626.0	731.7	573-065	1.00	1.00
573-069	632.3	401.2	573-069	0.50	2.00
573-072	509.5	690.7	573-072	2.00	1.00
573-074	787.0	829.8	573-074	0.50	0.75
573-077	508.0	469.2	573-077	0.75	0.50
573-091	504.9	514.2	573-091	1.00	0.50
573-094	678.3	568.4	573-094	0.75	0.25
573-099	468.3	644.2	573-099	2.00	0.75
Mean	**613.6**	**568.4**	**Median**	**0.75**	**0.75**
S.D.	**151.0**	**139.7**	**Range**	**(0.5 – 4.0)**	**(0.25 – 2.0)**

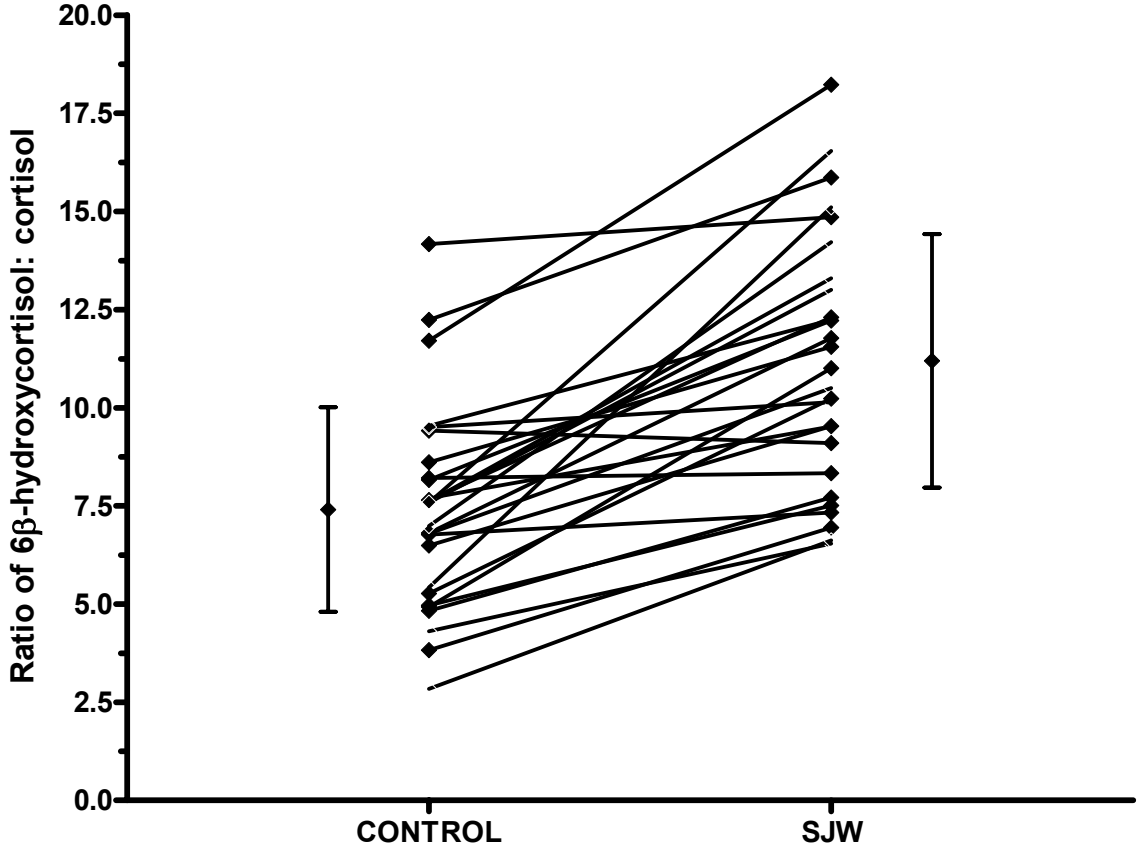


Figure 27 CYP3A induction as measured by the ratio of 6β-hydroxycortisol: cortisol in the presence (SJW) and absence (Control) of St. John's wort extract, 300 mg for fourteen days.

APPENDIX C

Individual Subject Data for Rosiglitazone-Trimethoprim and Rosiglitazone-St. John's wort Interaction Studies Sorted by Genotype

Table 22 Individual rosiglitazone pharmacokinetic parameters for subjects presenting with the *CYP2C8*1/*1* genotype

SN	AUC (μg*h/L)	SN	Cl/F (L/h)	SN	t1/2 (h)
573-003	3749	573-003	2.1	573-003	3.3
573-004	2759	573-004	2.9	573-004	4.2
573-005	3832	573-005	2.1	573-005	2.8
573-006	2879	573-006	2.8	573-006	3.3
573-009	2728	573-009	2.9	573-009	3.3
573-010	3893	573-010	2.1	573-010	5.5
573-013	2569	573-013	3.1	573-013	3.2
573-029	3331	573-029	2.4	573-029	3.2
573-034	4266	573-034	1.9	573-034	3.3
573-035	2998	573-035	2.7	573-035	4.8
RT2	3282	RT2	2.4	RT2	3.4
RT3	1987	RT3	4.0	RT3	3.2
RT5	2737	RT5	2.9	RT5	3.8
RT6	3288	RT6	2.4	RT6	4.4
Mean	**3164**	**Mean**	**2.6**	**Mean**	**3.7**
S.D.	**619**	**S.D.**	**0.6**	**S.D.**	**0.7**
SN	**Vd/F (L)**	**SN**	**Cmax (μg/L)**	**SN**	**tmax (h)**
573-003	10.2	573-003	703	573-003	0.75
573-004	17.7	573-004	501	573-004	0.75
573-005	8.6	573-005	885	573-005	0.50
573-006	13.2	573-006	683	573-006	0.50
573-009	14.0	573-009	562	573-009	0.50
573-010	16.2	573-010	759	573-010	0.50
573-013	14.2	573-013	476	573-013	2.00
573-029	11.1	573-029	694	573-029	0.50
573-034	9.0	573-034	910	573-034	0.50
573-035	18.4	573-035	651	573-035	0.75
RT2	11.8	RT2	767	RT2	0.50
RT3	18.3	RT3	661	RT3	0.50
RT5	16.1	RT5	381	RT5	4.00
RT6	15.3	RT6	697	RT6	0.50
Mean	**13.9**	**Mean**	**666**	**Median**	**0.50**
S.D.	**3.4**	**S.D.**	**148**	**Range**	**(0.5 – 4.0)**

Key: RT: Subjects who participated in rosiglitazone-trimethoprim interaction study

Table 23 Individual rosiglitazone pharmacokinetic parameters for subjects presenting with the *CYP2C8*1/*2* genotype

SN	AUC (μg*h/L)	SN	Cl/F (L/h)	SN	t1/2 (h)
573-025	3125	573-025	2.56	573-025	4.2
573-042	4417	573-042	1.81	573-042	4.8
573-062	3910	573-062	2.05	573-062	4.1
573-069	2682	573-069	2.98	573-069	3.8
RT4	2317	RT4	3.45	RT4	3.6
Mean	**3290**	**Mean**	**2.57**	**Mean**	**4.1**
S.D.	**866**	**S.D.**	**0.67**	**S.D.**	**0.4**
SN	**Vd/F (L)**	**SN**	**Cmax (μg/L)**	**SN**	**tmax (h)**
573-025	15.7	573-025	490	573-025	2.00
573-042	12.5	573-042	775	573-042	1.00
573-062	12.1	573-062	806	573-062	0.50
573-069	16.3	573-069	632	573-069	0.50
RT4	18.1	RT4	388	RT4	1.00
Mean	**15.0**	**Mean**	**618**	**Median**	**0.50**
S.D.	**2.6**	**S.D.**	**180**	**Range**	**(0.5 – 4.0)**

Key: RT: Subjects who participated in rosiglitazone-trimethoprim interaction study

Table 24 Individual rosiglitazone pharmacokinetic parameters for subjects presenting with the *CYP2C8*1/*3* genotype

SN	AUC (μg*h/L)	SN	Cl/F (L/h)	SN	t1/2 (h)
573-001	2968	573-001	2.70	573-001	4.0
573-011	2789	573-011	2.87	573-011	4.2
573-036	3561	573-036	2.25	573-036	5.4
573-038	2794	573-038	2.86	573-038	3.1
573-065	3535	573-065	2.26	573-065	3.4
573-091	2791	573-091	2.87	573-091	4.0
573-094	2320	573-094	3.45	573-094	2.5
573-099	2821	573-099	2.84	573-099	3.3
RT1	3387	RT1	2.36	RT1	2.7
RT7	3374	RT7	2.37	RT7	4.2
RT8	1931	RT8	4.14	RT8	3.2
Mean	**2934**	**Mean**	**2.81**	**Mean**	**3.6**
S.D.	**511**	**S.D.**	**0.57**	**S.D.**	**0.8**
SN	**Vd/F (L)**	**SN**	**Cmax (μg/L)**	**SN**	**tmax (h)**
573-001	15.7	573-001	652	573-001	0.75
573-011	17.5	573-011	816	573-011	0.50
573-036	17.5	573-036	546	573-036	0.75
573-038	12.9	573-038	574	573-038	0.75
573-065	11.0	573-065	626	573-065	1.00
573-091	16.6	573-091	505	573-091	1.00
573-094	12.7	573-094	678	573-094	0.75
573-099	13.4	573-099	468	573-099	2.00
RT1	9.0	RT1	1104	RT1	0.75
RT7	14.4	RT7	810	RT7	0.75
RT8	19.1	RT8	586	RT8	0.75
Mean	**14.5**	**Mean**	**670**	**Median**	**0.75**
S.D.	**3.1**	**S.D.**	**182**	**Range**	**(0.5 – 2.0)**

Key: RT: Subjects who participated in rosiglitazone-trimethoprim interaction study

Table 25 Individual rosiglitazone pharmacokinetic parameters for subjects presenting with the *CYP2C8*1/*4* genotype

SN	AUC (μg*h/L)	SN	Cl/F (L/h)	SN	t1/2 (h)
573-037	3020	573-037	2.65	573-037	4.4
573-046	3130	573-046	2.56	573-046	4.2
573-055	2358	573-055	3.39	573-055	3.2
573-072	3838	573-072	2.08	573-072	4.7
573-074	4155	573-074	1.93	573-074	4.4
573-077	2879	573-077	2.78	573-077	3.4
Mean	**3230**	**Mean**	**2.56**	**Mean**	**4.1**
S.D.	**658**	**S.D.**	**0.53**	**S.D.**	**0.6**
SN	**Vd/F (L)**	**SN**	**Cmax (μg/L)**	**SN**	**tmax (h)**
573-037	16.8	573-037	472	573-037	1.00
573-046	15.6	573-046	642	573-046	0.50
573-055	15.7	573-055	302	573-055	4.00
573-072	14.3	573-072	510	573-072	2.00
573-074	12.2	573-074	787	573-074	0.50
573-077	13.8	573-077	508	573-077	0.75
Mean	**14.7**	**Mean**	**537**	**Median**	**0.75**
S.D.	**1.6**	**S.D.**	**164**	**Range**	**(0.5 – 4.0)**

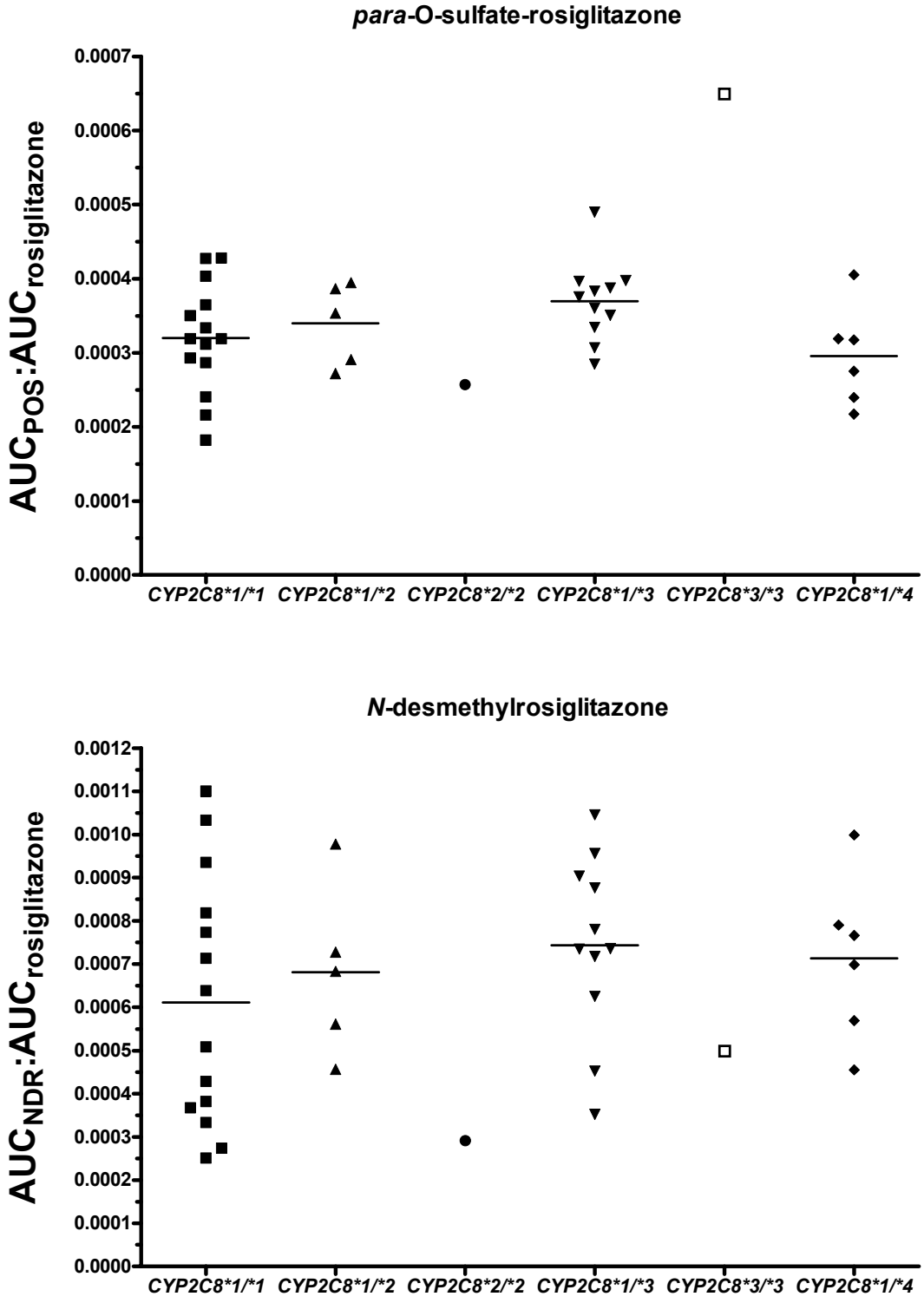


Figure 28 Formation rate of *para*-O-sulfate-rosiglitazone and *N*-desmethylrosiglitazone as measured by the metabolite AUC: rosiglitazone AUC.

APPENDIX D

Rosiglitazone-Trimethoprim Drug Interaction Study Protocol, Informed Consent, Advertisement, and Approval Letter

A. Principal Investigator: Reginald F. Frye, Pharm.D., Ph.D.
Co-Investigators: Matthew Hruska, Pharm.D., Janet Amico, M.D.

B. Protocol Title: Effect of CYP2C8 inhibition by trimethoprim on the pharmacokinetics of rosiglitazone.

C. Hypothesis and Specific Aims

The primary aim of this study is to test the hypothesis that the selective CYP2C8 inhibitor trimethoprim will decrease the clearance (increase the AUC) of rosiglitazone and decrease formation of its para-hydroxylated metabolite. We will accomplish this aim by characterizing the pharmacokinetics of rosiglitazone in the absence and presence of trimethoprim in healthy volunteers. A secondary, exploratory aim of this study is to describe rosiglitazone pharmacokinetics in individuals expressing the *CYP2C8*2* or *3 allelic variants.

D. Background

In humans, the cytochrome P450 (CYP) 2C subfamily, which includes the four isoenzymes 2C8, 2C9, 2C18, and 2C19, is responsible for the metabolism of several drugs including non-steroidal anti-inflammatory drugs, tolbutamide, phenytoin, omeprazole, and diazepam [1-3]. CYP2C8 is primarily distributed in the liver [3], and is involved in the metabolism of paclitaxel [4], retinoic acid [4], and rosiglitazone [5-7]. Two polymorphisms have been identified in the CYP2C8 enzyme: *CYP2C8*2* and *CYP2C8*3* [8]. The *CYP2C8*2* polymorphism is associated with a two-fold lower intrinsic clearance of paclitaxel and a decreased affinity for 6α-hydroxypaclitaxel, a major metabolite of paclitaxel; whereas *CYP2C8*3* is associated with decreased paclitaxel turnover [3, 8]. Allelic frequencies have been reported to be 0.18 for *CYP2C8*2* in African-Americans and 0.13 for *CYP2C8*3* in Caucasians [8]. Identification of these polymorphisms serves as a basis to investigate variability associated with CYP2C8 *in vivo* enzymatic activity.

Rosiglitazone is a thiazolidinedione antihyperglycemic agent used in the treatment of type 2 diabetes [6, 7]. Thiazolidinediones exert their clinical effects via the peroxisome proliferator-activated receptor γ (PPARγ) [9, 10], which facilitates the expression of genes responsible for glucose and lipid metabolism. Rosiglitazone has a half-life in humans of approximately 3 to 4 hours, a volume of distribution of approximately 18 liters, and it is 99.8% bound to plasma proteins. The maximum plasma concentration is reduced and the time at which the maximum concentration is achieved is delayed in the presence of food. Age and renal function exert no effect on rosiglitazone pharmacokinetics [9]. Rosiglitazone undergoes extensive metabolism with essentially no parent drug excreted unchanged in the urine. The two major metabolites of rosiglitazone isolated via microsomal and animal studies are *para*-hydroxy rosiglitazone and N-desmethyl rosiglitazone, both of which are produced by CYP2C8 [5, 6]. The sulfate conjugated *para*-hydroxy metabolite, *para*-O-sulfate-rosiglitazone, has been shown in humans to be the major metabolite isolated in plasma [7]. Based on these characteristics, conversion of rosiglitazone to the *para*-O-sulfate-rosiglitazone metabolite may be useful as an isoform specific index of CYP2C8 enzyme activity.

An important consideration when evaluating a potential probe drug is whether the probe suitably detects variation in activity of the target enzyme. Thus, it has been suggested that one means by which an enzyme-specific probe can be validated is to evaluate the pharmacokinetics of the probe drug in subjects who are treated with an inhibitor of the target enzyme [10]. The CYP inhibitor to be administered in this study is trimethoprim, an antibiotic predominantly used in the treatment of

respiratory and urinary tract infections. Trimethoprim possesses antimicrobial activity against such organisms as *Streptococcus pyogenes, Staphylococcus epidermis, Haemophilus influenzae, Escherichia coli, Proteus mirabilis,* and *Klebsiella spp.* The mechanism of action of trimethoprim is through competitive inhibition of the enzyme dihydrofolate reductase, a key enzyme in the folate pathway, which blocks DNA synthesis and leads to cellular death [11]. Basic pharmacokinetic parameters of trimethoprim include peak plasma concentrations occurring in 1-4 hours after single dose, bioavailability of 95%, $t_{1/2}$ of ~10 hours, protein binding of 44%, a volume of distribution ranging from 70 -100 L, and excretion primarily renally as unchanged drug [11-14]. Trimethoprim has been shown *in vitro* to selectively inhibit CYP2C8 at concentrations ranging from 5 to 100 μM (1.45 – 29 μg/ml), with 80% inhibition anticipated to occur at a concentration at the enzyme site of 20μM (5.8 μg/ml) [15]. Trimethoprim 200 mg administered twice daily reaches an average steady state (Css_{ave}) plasma concentration of 3.1 μg/ml [14]. The associated hepatic concentration (enzyme site), based on the liver to plasma partition of 6.5:1 determined in the rhesus monkey, is expected to be approximately 20 μg/ml, thereby yielding an anticipated inhibition of CYP2C8 greater than 80% [16].

The purpose of this study is to characterize the pharmacokinetics of rosiglitazone in the absence and presence of trimethoprim in healthy volunteers. In addition, we will begin to gather preliminary data on the effect of genetic variation in CYP2C8 on the pharmacokinetics of rosiglitazone. Due to the allele frequencies, we do not anticipate having an adequate number of individuals with allelic variants to determine the impact of the noted polymorphisms, but this will generate important preliminary data to support subsequent evaluation under a separate study.

E. Progress and Preliminary Studies

No studies to date have been performed.

F. Design and Methods

Design Overview: This study will be conducted in 8 subjects (4 men /4 women), who are 18 to 65 years of age, and will involve rosiglitazone administration as follows: (1) rosiglitazone 8 mg alone and (2) rosiglitazone 8 mg during trimethoprim 200 mg twice-daily. The order of administration will be randomized in a cross-over design to avoid any bias or sequence related effect. The visits will be separated by a washout period of one to two weeks.

Screening Visit: Subjects will be asked to read and sign the informed consent at the screening visit prior to the performance of any study-related procedures. The screening visit lasts approximately one (1) hour and 10 ml of blood will be withdrawn. Age, height, and weight will be recorded in a demographic record. To participate, the subjects must not have any evidence of abnormal renal function (i.e., creatinine clearance as estimated by the Cockroft-Gault equation < 80 ml/min) or hepatic function (i.e., ALT or AST greater than 2.0-times the upper limit of normal or total bilirubin out of the normal range). Women of childbearing potential will be given a pregnancy test to exclude pregnancy. Once subjects are determined eligible they will return to the GCRC for study visit 1.

Study Visit Procedures: Subjects will be asked to abstain from alcohol and caffeine containing foods and beverages for 24 hours and from grapefruit or grapefruit juice for 48 hours prior to each study visit. Subjects will also be asked to abstain from any over-the-counter medications, including non-steroidal anti-inflammatory drugs (e.g., naproxen and similar drugs), for 48 hours prior to each study visit. Subjects will report to the GCRC by 6 PM the evening prior to each rosiglitazone dosing day. They will fast from midnight the night before dosing until 2 hours (approximately 10:00 AM) after rosiglitazone administration. Prior to dosing, an intravenous catheter will be inserted into an arm

vein for the purpose of obtaining blood samples. At approximately 12:00 PM, subjects may eat a standardized lunch, and after approximately 4:00 PM subjects may eat per normal schedule. Standardized lunch and dinner meals will be provided while at the GCRC. The subjects may drink water as needed. All subjects will be genotyped for CYP2C8 allelic variants using a portion of the baseline blood sample collected for pharmacokinetic studies.

Rosiglitazone Pharmacokinetics: Blood samples (N=14; 10 ml or 2 teaspoons each, 140 ml total) will be obtained before and 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours after drug administration. Total voided urine will be collected via clean catch method before and at the following time intervals after the rosiglitazone administration: 0-8 hr and 8-24 hr. Blood samples will be drawn in EDTA tubes, kept on ice, and centrifuged within two hours of collection at 2800 rpm, 4^{o}C, for 15 minutes. Plasma will be harvested and stored at -70^{o}C or colder until analysis. Urine will be refrigerated throughout the study day. At the completion of each collection interval, the total volume of urine will be recorded and multiple aliquots will be stored at –70^{o}C or colder until analysis. Subjects will remain in the GCRC until the last blood draw; the total time required for subjects to remain in the GCRC is approximately 40 hours.

Study Visits

Rosiglitazone alone: Subjects will come to the General Clinical Research Center (GCRC) and will be given a single oral dose of rosiglitazone 8 mg at approximately 8 AM. Blood and urine collection will occur as described above. Subjects will be discharged after the last blood draw at 24 hours.

Rosiglitazone and trimethoprim: Subjects will be given five trimethoprim 200mg tablets to self-administer as trimethoprim 200 mg twice daily at approximately 7:30 AM and 7:30 PM for three days. On the third day, subjects will report to the GCRC by 6:00 PM, give a blood sample (10 ml) at approximately 7:00 PM, and will be given trimethoprim 200 mg at approximately 7:30 PM. Subjects will be administered 3 more doses of trimethoprim 200 mg every 12 hours for a total of ten doses (**Table 1**). We anticipate that trimethoprim 200mg twice daily will yield an average plasma concentration of 3.1 μg/ml. Subjects will then undergo the same procedure as in rosiglitazone alone with additional blood draws (10 ml) at 30, 36, and 48 hours after rosiglitazone dosing and additional urine collection from 24 to 36 hours. Subjects will be discharged after the last blood draw at 36 hours, but must return for a 48-hour blood sample; the total time required for subjects to remain in the GCRC is approximately 50 hours. A total of seventeen (17) blood samples (170 ml) will be obtained during this study period.

Table 1

Day	-3	-2	-1	1	2
Rosiglitazone 8 mg				X	
Trimethoprim 200 mg BID	X	X	X	X	X

A portion of the blood sample obtained prior to rosiglitazone dosing will be used for CYP2C8 genotyping, which will allow a preliminary comparison of para-O-rosiglitazone formation rate between the genotypes *CYP2C8*1/*2* and *CYP2C8*1/*3* versus wild type *CYP2C8*1/*1* .

The total amount of blood to be collected over the duration of the study, including screening labs, is approximately 320 milliliters (22 tablespoonfuls).

Sample Analysis

Rosiglitazone and *para*-O-rosiglitazone will be measured using high performance liquid chromatography (HPLC) with fluorescence detection based on a method reported by Cox et al [7]. Trimethoprim will be measured in plasma by an HPLC ultraviolet detection assay reported by Ronn et al [17].

Data Analysis

Rosiglitazone. The rosiglitazone concentration-time data will be analyzed by noncompartmental methods. The terminal elimination rate constant (λz) will be estimated by nonlinear least squares regression analysis of the terminal portion of the concentration-time data. Apparent elimination half-life will be calculated as $0.693/ \lambda z$. The area under the concentration time curve (AUC) and the area under the first moment of the concentration time curve (AUMC) will be calculated by the linear trapezoidal rule with extrapolation to infinity. Mean residence time (MRT) will be calculated as [$AUMC_{0-\infty}$ / $AUC_{0-\infty}$]. Apparent oral clearance (CL_O) will be determined as [Dose / $AUC_{0-\infty}$] and the apparent steady-state volume of distribution will be determined as [$CL_O \times MRT$]. The formation clearance of *para*-O-rosiglitazone will be calculated as the amount of each metabolite recovered in the urine, divided by the plasma rosiglitazone AUC from the same time interval.

Trimethoprim. The trimethoprim concentration-time data will be analyzed by non-compartmental methods. The area under the concentration time curve (AUC) will be calculated by the linear trapezoidal rule over the duration of rosiglitazone blood sampling.

CYP2C8 Genotyping. CYP2C8 genotype data will be used to generate preliminary data on the effect of CYP2C8 allelic variants on rosiglitazone pharmacokinetics. The DNA obtained from a single blood sample will be stored in Dr. Frye's laboratory and will not be made available to anyone for purposes other than those of this study. Samples will be stored without personal identifiers, using a unique study code, and destroyed within one year after the conclusion of this study. Subjects may request at any time that their samples be destroyed or released to a designee of their choosing, but only with written permission. Samples will be destroyed within one week.

G. Biostatistical Design and Analysis

Sample Size and Power: The objective of this study is to determine whether rosiglitazone clearance and *para*-hydroxylation are inhibited in the presence of trimethoprim. This study is a randomized two-way, crossover design and the primary endpoints are rosiglitazone AUC and para-O-rosiglitazone formation clearance, which serve as surrogate estimates of CYP2C8 activity. Subjects will be randomized by randomly permuted blocks (8 subjects randomized in 2 blocks). There will not be any stratifying factors employed. Sample size calculations were performed using PASS (version 6.0, Kaysville, UT) and are based on the within-subject coefficient of variation (CV) reported for rosiglitazone AUC, which was 18% [18]. Using this within-subject CV, the proposed sample size of N = 8 subjects will allow for the detection of an effect size of 1.5 (90% power, α = 0.05, two-sided), which translates into a 27% difference. The expected magnitude of difference is more than 100%.

Statistical Analysis Plan: Rosiglitazone AUC and *para*-O-rosiglitazone formation clearance will be compared by paired t-test. Parameter values will be log-transformed prior to analysis since these values exhibit log-normal distribution. A two-sided $p \leq 0.05$ will be considered significant and all calculations will be performed using SAS software version 8.02 (SAS, Cary, NC). The expected outcomes are that rosiglitazone AUC and para-O-rosiglitazone formation clearance, surrogate

markers of CYP2C8 activity, will be decreased in the presence of trimethoprim as a result of enzyme inhibition.

H. Human Subjects

1. Subject Population. Eight (8) healthy subjects aged between 18 and 65 years will complete this study. Based on past experience, we anticipate a screening failure rate of approximately 25%, thus the proposed number of subjects enrolled per IRB guidelines will be ten (10), which allows for potential dropouts. An even number of men and women will be recruited and will give written informed consent. As defined by NIH, this study will enroll children aged 18 to 21 years; children less than 18 years of age will be excluded from study participation since this study poses greater than minimal risk and there is no potential for direct benefit to the subject.

Each subject will have to pass a screening evaluation based on history, physical examination and the following biochemical and urinalysis tests: BUN, creatinine, electrolytes, liver function tests, total protein, albumin, complete blood count, urine pH, hemoglobin, protein, glucose, specific gravity and microscopic examination of sediment. Pregnant women, or women who are currently breast-feeding an infant, will not be allowed to take part in this study. Women of childbearing potential will be tested to exclude pregnancy using a urine pregnancy test. Use of oral contraceptives or hormonal replacement therapy will be permitted. Abstinence from alcohol (in any form) and caffeine for 24 hours and grapefruit/grapefruit juice for 48 hours before and during the period of the rosiglitazone pharmacokinetic studies is also required.

Minority Inclusion Statement: Women or men of all races will be eligible and recruited for this study. Women and men will be balanced. The racial and age mix of the study will be representative of the population of Western Pennsylvania. There will be no exclusion based on race, sex, or ethnicity.

Total Planned Enrollment: 8

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	**Sex/Gender**		
	Females	**Males**	**Total**
Hispanic or Latino	0	0	0
Not Hispanic or Latino	4	4	8
Ethnic Category Total of All Subjects*	4	4	8
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	1	1	2
White	3	3	6
Racial Categories: Total of All Subjects *	4	4	8

Inclusion Criteria

1. Non-smokers (self-reported) aged between 18 and 65 years.
2. Body Mass Index (BMI) < 31.
3. Signed Informed Consent.

Exclusion Criteria

1. Any evidence of renal dysfunction (estimated creatinine clearance < 80ml/min).
2. Any evidence of impaired hepatic function (liver enzymes greater than two times the upper limit of normal or bilirubin outside the normal range).
3. Taking any medications other than oral contraceptives or hormonal replacement therapy.
4. Women who are pregnant or are currently breastfeeding.
5. Prior hypersensitivity to trimethoprim and/or co-trimoxazole (i.e. Bactrim or Septra)

2. Sources of Research Material: During this study, thirty-one blood samples (310 ml) will be collected during the pharmacokinetics studies. A portion of a single blood sample will be used to obtain DNA for genotyping. Urine will be collected in intervals for 24-36 hours after rosiglitazone administration. The blood and urine will be obtained specifically for research purposes in order to meet our specific aim.

3. Recruitment Methods and Consent Procedures: Subjects will be recruited through an advertisement that will be placed in the Pitt News (See Appendix A). Informed Consent will be obtained prior to any screening procedures. Subjects considering enrollment in the study will be provided with an IRB approved consent form to read, and the study protocol will be explained to them. Any questions that potential subjects have will be answered by a physician co-investigator; if interested in study participation subjects will be required to sign the consent form and have that witnessed. A copy will be maintained in the subject's GCRC chart and a copy retained by the investigator. A physician co-investigator will obtain consent and sign the Investigator's Certification. Eligible subjects will then undergo a screening assessment based on history, physical examination and laboratory tests to assess their suitability for the study.

4. Potential Risks: Side effects are listed below for each of the drugs used in this study. These medications may cause all, some, or none of the side effects listed below. Furthermore, there is always the risk of very uncommon or previously unknown side effects occurring. Side effects that are considered *likely* occur in more than 10 out of every 100 (10%) people who take the drug, *common* side effects occur in approximately 1 to 10% of people, and *rare* side effects occur in less than 1% of people.

Rosiglitazone has been well-tolerated in several drug interaction studies conducted in healthy volunteers [18-21]. Unlike other anti-diabetic agents, rosiglitazone has not been shown to cause hypoglycemia in healthy volunteers. Rosiglitazone has been shown to be associated with hepatocellular injury in men greater than 60 years of age after 2 to 3 weeks of daily therapy.

Rosiglitazone (8 mg) is a FDA approved anti-diabetic agent used alone or in combination in the treatment of Type 2 diabetes mellitus.
Likely: Weight gain.
Common: Upper respiratory tract infection, headache, peripheral edema, back pain or injury, fatigue, hyperglycemia, diarrhea, and anemia.
Rare: Exacerbation of CHF, elevated transaminases, hepatic failure, hepatitis, increased bilirubin, pulmonary edema.

Trimethoprim, 200 mg twice daily, has been administered safely for five days in the treatment of urinary tract infection and yielded a lower incidence of adverse effects versus the sulfamethoxazole/trimethoprim combination product (e.g., Co-trimoxazole). The most frequent events reported were gastrointestinal disturbances such as nausea, vomiting, and diarrhea [22]. Healthy volunteers tolerated doses of 12 mg/kg/day and reported nausea and headaches as the

most frequent adverse events [23]. Large doses up to 23 mg/kg/day have been utilized in the treatment of *Pneumocystis carinii* Pneumonia (PCP) in AIDS patients, with the most frequent adverse events reported as gastrointestinal disturbances, followed by LFT abnormalities, neutropenia, and thrombocytopenia [24, 25]. The frequency of these events increases with the duration of therapy and sustained plasma concentrations greater than 5 μg/ml [25]**.** It is anticipated in this study that subjects administered 200 mg twice daily for 5 days will achieve steady-state concentrations of 3.0 μg/ml and maximum concentrations of ~4.0 μg/ml, which are below the threshold of 5 μg/ml [14].

Trimethoprim (200 mg) is an FDA approved antibiotic used in the treatment of infections of the urinary and respiratory tracts.
Likely: None.
Common: Rash, pruritis, megablastic anemia (with chronic high dose).
Rare: Fever, exfoliative dermatitis, nausea, vomiting, epigastric distress, thrombocytopenia, neutropenia, leukopenia, hyperkalemia, cholestatic jaundice, increased LFTs, elevated BUN/serum creatinine

The risks involved with this study include the discomfort and inconvenience of having an intravenous catheter placed and blood samples collected with potential for pain, bruising, bleeding and infection.

Due to the genetic component of this study, there is a risk for breach of confidentiality, which could impact future insurability, employability or reproduction plans, or have a negative impact on family relationships, and/or result in paternity suits or stigmatization. The DNA obtained will be stored in Dr. Frye's laboratory and will not be made available to anyone for purposes other than those of this study. Samples will be stored without personal identifiers, using a unique study code, and destroyed in one year after the conclusion of this study. Subjects may request at any time that their samples be destroyed or released to a designee of their choosing, but only with written permission.

5 Risk Management Procedures: The risk of adverse experiences in this study will be minimized by utilizing only qualified individuals to conduct the study, the staff in GCRC in UPMC-MUH. Appropriate attention to detail in the experimental setting will be emphasized. Moreover, this study will use small, single doses of rosiglitazone and short-term exposure to trimethoprim and thus, the likelihood of dose-related adverse events should be minimized. Immediate medical treatment will be provided for any illness or injury resulting from this study. Trained nursing staff are present in the GCRC at all times and the physician co-investigator will also be available to evaluate the subject. In the event that a subject experiences an intolerable side effect, the subject will be withdrawn from the study and followed for resolution of the effect(s). A subject may also be removed from the study if in the opinion of the physician investigator, it is in the subject's best interest.

Data Safety Monitoring Plan. This study involves a small number of subjects who will be closely monitored by the Investigators and research personnel on the General Clinical Research Unit. The data and safety information obtained in each study subject is reviewed at a weekly or biweekly meeting held by the Investigators. As a part of these meetings, the research team will monitor data, confidentiality, and recruitment in addition to adverse events. A summary report from the meetings will be submitted to the IRB at the time of annual renewal. If a serious life-threatening event occurs, the event will be reported immediately (i.e., within 24 hours) to both the FDA and the IRB. Unexpected reactions of moderate or greater severity will be reported to

the IRB within 10 calendar days of the reaction. Minor events will be reported to the IRB at the time of annual review.

6. **Evaluation of the Risk/Benefit Ratio:** No personal benefit will result from this study; however, this study may provide important information on the use of rosiglitazone as a CYP2C8 enzyme selective probe drug. The risk to benefit ratio of this study is considered low.

7. **Cost and Payment:** All procedures necessary for the completion of these studies will be performed at no cost to the study subjects (or their insurance providers). The subjects will be paid for study participation. Subjects will be paid $500 for completion of the entire study. If only one of the two study visits is completed, subjects will be paid $200. Other than the payment and their contribution to our knowledge of drug action, there are no benefits to the subjects for participating in this study.

I. Justification for Utilization of GCRC Resources

The GCRC use is being requested to take advantage of the facilities and expertise available there to ensure proper conduct of the study. It will ensure that drugs are given under medical supervision and samples are collected by qualified professionals with experience in the conduct of research studies. These factors are important to obtain results that are valid and interpretable.

J. Study Size and GCRC Resources

1.	Number of subjects	10 (8 to complete)
2.	Annual number of research patient days	32
3.	Annual number of outpatient visits	18 (screening, 8 outpatient blood draws)
4.	Estimated Ancillary Costs Per Patient Days	
5.	Estimated Ancillary Costs Per Outpatient Visit	
6.	Description of Other GCRC Resources Required	None

K. Research needs to be provided by Investigator's laboratory

Analytical assays required for the determination of concentrations of rosiglitazone, *para*-O-rosiglitazone, and trimethoprim will be conducted by Dr. Frye. Labeled storage containers for plasma/urine samples will be provided.

L. Funding Support: Pharmacodynamic Research Center

M. References

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N. Qualifications of Investigators

Reginald F. Frye, Pharm.D., Ph.D. is an Assistant Professor in the Department of Pharmaceutical Sciences, School of Pharmacy, and a member of the Center for Clinical Pharmacology. He is also Director of the Clinical Pharmacology Analytical Facility. He has expertise in the evaluation of drug metabolism in humans.

Matthew W. Hruska, Pharm.D, is a Ph.D. student in the Clinical Scientist Program in the School of Pharmacy and has experience in the conduction of clinical research studies. He will serve as the study coordinator.

Janet A. Amico, M.D. is a Professor of Pharmaceutical Sciences and Medicine in the Department of Pharmaceutical Sciences and Division of Endocrinology. She is active in both clinical and research endeavors that focus on endocrinology.

PROTOCOL APPENDIX A

ADVERTISEMENT

Healthy men and women 18-65 years old are needed for a research study
evaluating how one drug affects the break down of another drug.
The study requires two 2-night stays (1 or 2 weeks apart) in UPMC-Montefiore.
Participants will be paid $500.00 upon study completion. Contact Matthew Hruska, School of Pharmacy. mwhst12@pitt.edu or 412-624-8105.

University of Pittsburgh

School of Pharmacy
Department of Pharmaceutical Sciences

904 Salk Hall
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412-624-3330
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Approval Date: 11/18/2002
Renewal Date: 11/17/2003
University of Pittsburgh
IRB#020971
Consent version: 11/14/2002

CONSENT TO ACT AS A SUBJECT IN AN EXPERIMENTAL STUDY

TITLE: Effect of CYP2C8 inhibition by trimethoprim on the pharmacokinetics of rosiglitazone.

Principal Investigator: Reginald F. Frye, Pharm.D., Ph.D.
Associate Professor, School of Pharmacy
807 Salk Hall
(412) 624-4683

Co-Investigators: Janet A. Amico, M.D.
Matthew W. Hruska, Pharm D
School of Pharmacy
904 Salk Hall
412-648-8430

SOURCE OF SUPPORT: Pharmacodynamic Research Center

TO PERSONS BEING ASKED TO PARTICIPATE IN THIS STUDY:
The following information is provided to inform you about a research study that you are being asked to participate in. Please read this form carefully. Any questions you may have about this study will be answered. Please feel free to ask any questions you may have about this study and/or about the information given below.

Why is this research being done?

You are being asked to participate in this study because you are a healthy man or woman between the ages of 18 and 65 years old. The purpose of this study is to determine if the antibiotic trimethoprim changes how the body metabolizes (breaksdown) the drug rosiglitazone. If you agree to participate in this study, you will receive on two separate occasions, a single doses of rosiglitazone 8 mg (a drug normally used to assist the body with adjusting sugar in the blood). Rosiglitazone is taken by mouth as a tablet to treat diabetes and has been approved for use by the US Food and Drug Administration (FDA). In addition, you will be asked to take an antibiotic twice a day for 5 days. You will take it at home for 3 of the days. The antibiotic you will be given is trimethoprim, 200 mg twice daily, an FDA approved antibiotic used in the treatment of respiratory and urinary tract infections. Both rosiglitazone and trimethoprim will be given by mouth.

 Subject Initials _____

Who is being asked to take part in this research study?

You are being invited to take part in this research study because you are a healthy volunteer. A total of up to ten healthy men and women will participate in this study. This study will require two (2) two-night stays in the General Clinical Research Center (GCRC) in UPMC-Montefiore Hospital. One visit will last for approximately 40 hours and the other visit will last for approximately 50 hours.

What procedures will be performed for research purposes?

If you agree to be one of the ten study subjects, you understand that the following will be required of you:

- You will have a physical exam and laboratory tests (requiring 10 mL or two teaspoonfuls of blood) to determine if you are eligible to participate in the study. The purpose of the blood sample is for the screening laboratory tests (to check how your kidney and liver are working). The exam and laboratory tests will be done within four (4) weeks prior to the start of the study. These examinations and screenings will require one to two hours of your time and are not designed for the purpose for your personal health care, but only for qualification to enter the study.
- If you are a woman of childbearing age, you will have a pregnancy test. If this is positive, you will not be eligible to participate in this study. Pregnant women, or women who are currently breast-feeding an infant, will not be allowed to take part in this study.
- To be eligible to participate in this study, you should not be taking any medications other than birth control pills or hormone replacement therapy. You will not be permitted to drink any alcoholic or caffeine containing food or beverages for 24 hours prior to the study visits. You will not have any grapefruit or grapefruit juice 48 hours prior to or during the study. You will be asked to abstain from taking any over the counter medications 48 hours prior to the study day and throughout your participation in this study (requiring an additional 50 hours).

<u>Experimental Procedures:</u>

If you qualify to take part in this research study, you will undergo the following experimental procedures:

1. There will be two overnight study visits at the GCRC. On one visit you will be given rosiglitazone alone and on the other visit you will be given trimethoprim and rosiglitazone. The second study visit will occur at either one (1) or two (2) weeks after the first study visit. The order in which these visits occur will be assigned by chance (like flipping a coin).
2. For the rosiglitazone alone study visit, you will report to the GCRC by 6:00 PM, the night prior to rosiglitazone administration. If you are female and capable of bearing children, a urine pregnancy test will be performed. If the pregnancy test is positive, you will not be allowed to participate in this study. The next morning, a catheter (thin plastic tube inserted into a vein) will be placed in a forearm vein of one of your arms for the purpose of blood collection. You will give a pre-dose blood sample (10 ml or two teaspoonfuls) from your catheter. At approximately 8:00 AM, you will be given a rosiglitazone tablet (8 mg) by mouth with 8 ounces of water. A 10 ml blood sample (two teaspoonfuls) will be obtained at 15, 30, and 45 minutes, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours after rosiglitazone administration. You will be allowed to eat regularly, with the exception of caffeine, two hours after you take rosiglitazone. You will remain in the GCRC overnight. Total voided urine will be collected before and at the following time intervals after rosiglitazone administration: 0-8 hr and 8-24 hr. The catheter will be removed after the 24-

hour blood sample is collected. You may leave the GCRC after collection of the 24-hour blood and urine samples.

3. For the rosiglitazone and trimethoprim study visit, you will be given five (5) trimethoprim 200 mg tablets to take at home. You will take one (1) trimethoprim 200 mg tablet twice daily, at approximately 7:30 AM and 7:30 PM, with a full glass of water for 3 days. A study coordinator will contact you to ask about taking the study drugs and to ask you if you are experiencing any side effects from the drug. On the evening of the third day of taking trimethoprim, you will report to the GCRC by 6:00 PM and receive another dose of trimethoprim at approximately 7:30 PM and continue to be given trimethoprim every 12 hours for a total of five (5) more times. You will be given rosiglitazone 8 mg at approximately at 8:30 AM. The blood draws will follow the same procedures as above with additional blood samples taken at 30, 36, and 48 hours and additional urine collection from 24-36 hours after taking rosiglitazone. You may leave the GCRC after collection of the 36-hour blood sample, but must return to the GCRC at 48 hours after rosiglitazone administration and give a 10 ml blood sample to measure the concentration of rosiglitazone.
4. A portion of one of the blood samples obtained will be collected to obtain deoxyribonucleic acid or DNA (genetic material). The DNA will be used to screen for genetic differences in drug metabolizing enzymes that break down drugs. The activities of these enzymes vary between individuals due to both genetic and environmental factors. The DNA obtained will be stored in the Principal Investigator's Laboratory in Salk Hall for use in the current study only. The samples will contain only a study code number and not your name or any other information identifying you. You can request at any time that the samples be destroyed. The blood samples will be under the control of the principal investigator listed on the first page of this form, and will be destroyed in one (1) year, at the conclusion of the study.
5. A total of thirty-one (31) blood samples will be obtained during the rosiglitazone study days for a total amount of 310 ml (about 10 ounces or 21 tablespoonfuls).
6. The total blood withdrawn from you during the study, including screening labs, will be approximately 22 tablespoonfuls or 320 ml. The amount of blood withdrawn when someone donates a unit of blood is approximately 30 tablespoonfuls or 450 ml.

What are the possible risks, side effects, and discomforts of this research study?

As with any investigational study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life-threatening. You will not experience any personal benefits to your health as a result of your participation in this study. You will receive a total of two (2) doses of rosiglitazone and ten (10) doses of trimethoprim for this study and that they may cause all, some, or none of the side effects listed below. You may experience previously unknown side effects or you may have an allergic reaction to the drug. You will be asked if you have received the study drugs in the past and, specifically, if you have experienced any side effects. If you have, you may not be able to participate in this study. If you are a woman capable of becoming pregnant, a pregnancy test will be conducted before you take the study drug. If you are pregnant, you will not be allowed to continue with this study. If you think you are experiencing a side effect from rosiglitazone or trimethoprim, you must let the research nurse at the GCRC and one of the investigators know immediately.

Side effects are listed below for each of the drugs used in this study. Side effects that are considered LIKELY, occur in more than 10% of people (more than 10 out of every 100 people) who take the drug,

COMMON side effects occur in approximately 1% to 10% of people (1 to 10 out of 100 people), and RARE side effects occur in less than 1% of people (less than 1 in 100 people).

Risks of the study drugs.

Rosiglitazone (8 mg) is an FDA approved drug used to lower blood sugar to treat Type 2 diabetes. Rosiglitazone has not been shown to cause hypoglycemia (low blood sugar, symptoms include sweating, lightheadedness, weakness, nausea, confusion or loss of consciousness) in healthy volunteers.
Likely (occurs in more than 10 out of every 100 people): Weight gain (usually seen with chronic use).
Common (occurs in approximately 1 to 10 out of every 100 people): Upper respiratory tract infection (fever, stuffy or runny nose), headache, peripheral edema (swelling of the feet or legs), back pain, injury, high blood sugar, diarrhea, anemia (decreased red blood cells; symptoms include dizziness, lightheadedness).
Rare (occurs in less than 1 out of every 100 people): Worsening of congestive heart failure, elevated liver enzymes, liver failure, hepatitis, increased bilirubin (a pigment released when the liver processes waste), pulmonary edema (increased fluid in the lungs).

Trimethoprim (200 mg) is an FDA approved antibiotic used to treat urinary and respiratory tract infections.
Likely (occurs in more than 10 out of every 100 people): None.
Common (occurs in approximately 1 to 10 out of every 100 people): Rash, itchiness, and with chronic high doses, megaloblastic anemia (large red blood cells, symptoms include dizziness, lightheadedness).
Rare (occurs in less than 1 out of every 100 people): (usually occur with duration of treatment greater than 10 days) Fever, exfoliative dermatitis (inflamed skin, which falls off in scales or layers), nausea, vomiting, epigastric distress (gastrointestinal discomfort such as epigastric pain (heartburn-like feeling), increased blood potassium levels, yellowing of the skin, elevated liver enzyme levels associated with liver failure, elevated blood urea nitrogen/serum creatinine (breakdown products of blood, muscle, and protein), neutropenia (low neutrophil counts), leukopenia (low leukocyte counts), thrombocytopenia (a decrease in platelet count), and abnormal liver function tests (an increase in the liver enzymes that is associated with liver damage). Neutrophils and leukocytes are white blood cells that fight infections. Platelets are cells responsible for blood clotting.

To avoid risk to the fetus, it is important that you not be pregnant when we conduct this study. We also advise that you (or your female sexual partner) not become pregnant for one week after ingestion of the study drugs. Avoiding sexual activity is the only certain method to prevent pregnancy. However, if you choose to be sexually active, you should use an appropriate "double barrier" method of birth control (such as female use of a diaphragm, intrauterine device (IUD), or contraceptive sponge, in addition to male use of a condom) or the female should be using prescribed "birth control" pills, injections, or implants. If you choose to be sexually active during this study, pregnancy could still result even with the use of these birth control methods. The risks of receiving the study drug while pregnant include potential loss of pregnancy or possible birth defects.

Risks of the Blood Tests:
Bruising, soreness, or rarely, infection may occur as a result of the needle sticks to obtain blood from your vein.

Risks of the Genetic Tests:
There is a possibility that if the results of a research study involving genetic material were to become generally known, this information could affects one's ability to be insured, employed, future plans for

children, or family relationships. In this study, all genetic data are stored without personal identifying information (all samples are stored with a unique study code number).

What are possible benefits from taking part in this study?
You will receive no direct benefit from taking part in this research study. Information from the DNA contained in the blood sample will not provide any immediate benefit to you. Since there are no established links between the information obtained and possible genetic changes, you will not be informed of the results of any gene testing. The DNA obtained will be stored in Dr. Frye's laboratory in Salk Hall for use in the current study only. The samples will be stored indefinitely for use in the current study only. You can request at any time that the samples be destroyed.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?
You will be promptly notified if any new information develops during the course of this study which may cause you to change your mind about continuing to participate.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?
Neither you, nor your insurance provider, will be charged for the costs of any of the procedures performed for the purpose of this research study (i.e., the Screening Procedures and Experimental Procedures described above).

Will I be paid if I take part in this research study?
Participation in this study is voluntary. If you decide to participate in this study, all procedures performed and medications given will be at no cost to you. Upon successful completion of the requirements of the study, you will be paid the sum of $500 for your participation. If, for whatever reason, you only complete the first of the two study visits, you will be paid $200. Parking fees related to your participation in this study will not be paid for by the study.

Your blood sample and its DNA used in this research study may contribute to a new invention or discovery. In some instances, these inventions or discoveries may be of commercial value and may be sold, patented, or licensed by the investigators and the University of Pittsburgh for use in other research or the development of new products. If you agree to participate in this research study, there are currently no plans to share with you any money that may result from the development of such products. You retain the right to have your blood samples and its DNA destroyed should you decide to withdraw from this research study.

Who will pay if I am injured as a result of taking part in this study?

University of Pittsburgh researchers and their associates who provide services at the UPMC Health System (UPMC HS) recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of the UPMC HS. It is possible that the UPMC HS may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment,

you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. You will not receive any monetary payment for, or associated with, any injury that you suffer in relation to this research.

Who will know about my participation in this research study?

All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept separate from the research records. Only the researchers listed on the first page of this form and their staff will have access to your research records. Initials and a code number will indicate your identity on donated blood samples and their DNA. University policy requires that all research records be kept for a period of not less than 5 years after the study ends.

Any information about you obtained from this research will be kept as confidential (private) as possible. You will not be identified by name in any publication of research results unless you sign a separate form giving your permission (release). In unusual cases, your research records may be released in response to an order from a court of law. It is also possible that authorized representatives of the Food and Drug Administration and/or the University Research Conduct and Compliance Office may inspect your research records. If the researchers learn that you or someone with whom you are involved is in serious danger or harm, they will need to inform the appropriate agencies as required by Pennsylvania law.

Is my participation in this research study voluntary?

Your participation in this research study is completely voluntary. You do not have to take part in this research study and, should you change your mind, you can withdraw from the study at any time. Your current and future care at a UPMC HS facility and any other benefits for which you qualify will be the same whether you participate in this study or not.

If I agree to take part in this research study, can I be removed from the study without my consent?

It is possible that you may be removed from the research study by the researchers if, for example, your pregnancy test proves to be positive, you experience a side effect that is intolerable, or if in the opinion of the physician investigator, it is in your best interest to no longer participate in this study.

Subject Initials _____

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IF I HAVE A QUESTION: If at any time in the study I wish to contact and discuss any aspect of the research study with the investigators I may contact Dr. Frye at (412) 624-4683 or any of the other investigators at the phone numbers provided on the front of this Consent Form.

VOLUNTARY CONSENT:

I certify that I have read the preceding, or it has been read to me, and I understand its contents. Any questions I have pertaining to the research have been, and will continue to be answered by the investigators listed at the beginning of this consent form at the numbers given. Any questions I have concerning my rights as a research subject will be answered by the Human Subjects Protection Advocate at the University of Pittsburgh IRB Office (412-578-8570). A copy of this consent form will be given to me. My signature below means I have freely agreed to participate in this project.

_____ _____
Subject Signature Date and Time

_____ _____
Witness Date and Time

I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participating in this research study, have answered any questions that have been raised, and have witnessed the above signature.

_____ _____
Investigator Signature Date and Time

Subject Initials _____

University of Pittsburgh

Institutional Review Board

3500 Fifth Avenue
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Pittsburgh, PA 15213
(412) 578-3424
(412) 578-8553 (fax)

MEMORANDUM

TO: Reginald Frye, Pharm.D., Ph.D.

FROM: Robert Sweet, M.D., Vice Chair *Sweet/ay*

DATE: November 18, 2002

SUBJECT: IRB #020971: Effect of CYP2C8 Inhibition by Trimethoprim on the Pharmacokinetics of Rosiglitazone

Thank you for addressing the concerns of the Institutional Review Board regarding the above-referenced proposal. This version of your protocol and consent form(s) has been approved by Subcommittee D.

Please include the following information in the upper right-hand corner of all pages of the consent forms:

Approval Date: November 18, 2002
Renewal Date: November 17, 2003
University of Pittsburgh
IRB #020971

Adverse events which occur during the course of the research study must be reported to the IRB Office. Please call the IRB Adverse Event Coordinator at 412-578-8569 for the current policy and forms.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month prior** to the expiration date noted above for annual renewal as required by Assurance No. IORG0000196, given to DHHS by the University of Pittsburgh.

If your research proposal involves an investigational drug, please forward a copy of this approval letter along with a copy of the Cover Sheet, protocol, consent form(s) and drug brochure to Investigational Drug Service, PUH Pharmacy.

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

RS:ay

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