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FACTORS AFFECTING CYP2C9-MEDIATED METABOLISM

James Matthew Hutzler

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Submitted to
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at
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In
Basic Pharmaceutical Sciences

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Keywords: cytochrome P450 2C9, flurbiprofen, naproxen, piroxicam, dapsone, atypical kinetics

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ABSTRACT

FACTORS AFFECTING CYP2C9-MEDIATED METABOLISM

James Matthew Hutzler

Cytochrome P450 enzymes can exhibit non-Michaelis-Menten, or atypical kinetics. In particular, dapsone activates CYP2C9-mediated flurbiprofen 4'-hydroxylation. However, the mechanism of activation of CYP2C9 is poorly understood. The activation of flurbiprofen, naproxen, and piroxicam metabolism by dapsone was investigated, fitting kinetic data to a two-site effector model, indicating that the CYP2C9 active site may have multiple binding regions. In addition the structural requirements for activation of CYP2C9 were explored using structural analogs of dapsone, with results suggesting that a sulfone group between two benzene rings is necessary for activation. CYP2C9 *2(R144C) and *3(I359L) allelic variants and the F114L mutant enzymes have also been used to see if these amino acid changes alter dapsone activation of CYP2C9. In this study, flurbiprofen 4'-hydroxylation was activated ~5-fold in the F114L mutant, but only ~2-fold for wild-type enzyme in the presence of dapsone. Also, piroxicam 5'-hydroxylation was not activated by dapsone in the F114L mutant, which had higher piroxicam hydroxylase activity than CYP2C9 wild-type, suggesting a role for F114 in activation. In addition, substrate metabolism was studied in CYP2C9/19 chimeric and CYP2C19 mutant enzymes. Results suggest that amino acids 228-292 are important for catalysis of flurbiprofen, naproxen, and piroxicam. In addition, the CYP2C19 KSN mutant has the greatest activity towards flurbiprofen 4'-hydroxylation, indicating that changes at residues 241, 286, and 289 confer activity to CYP2C19. It has also been discovered that hydroxy-dapsone, the major phase I metabolite of dapsone activates flurbiprofen metabolism, although through a different mechanism. Spectral binding studies confirm kinetic data, indicating that dapsone directly affects the binding of flurbiprofen, whereas hydroxy-dapsone does not. Additional studies with ibuprofen indicates that dapsone alters the binding orientation of this substrate, as the ratio of 3-OH to 2-OH ibuprofen changes in the presence of dapsone. Lastly a urine and plasma assay for detection of flurbiprofen and 4'-hydroxy flurbiprofen was developed for use in analysis of samples in a clinical study investigating activation of metabolism *in vivo*. It was found that dapsone activates flurbiprofen 4'-hydroxylation *in vivo* about 10%, much less than that observed *in vitro*.

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CHAPTER 1

Literature Review

1.1. Introduction to Atypical Kinetics

It is well established that the cytochrome P450 monooxygenase family of enzymes are the most important enzymes involved in the biotransformation of both xenobiotic and endobiotic hydrophobic compounds [1;2]. The cytochrome P450 enzymes most often play a protective role against foreign chemicals, although they are also implicated in the bioactivation of certain procarcinogens [e.g. benzo[a]pyrene], and in many metabolism-based drug-drug interactions in patients taking concomitant medications. Consequently, it is the goal of drug metabolism and toxicology labs to not only determine CYP450 isoform contribution for the metabolism of a given compound, but also to understand the various factors that affect the activity and behavior of these isoforms. Classically, the metabolism of a particular compound is described kinetically using the Michaelis-Menten equation, which yields estimates of maximal reaction velocity ($V_{\max}$) and substrate affinity (K_m). However, it is becoming increasingly evident that the kinetic profile of the metabolism of some drugs may be better described by a non-Michaelis-Menten, or atypical kinetic model, complicating interpretation of data. Examples of these atypical kinetic profiles have become more numerous over the past several years and are proposed to fall into five categories: activation, autoactivation, substrate inhibition, partial inhibition, and biphasic metabolism [3]. Activation is evident when P450 enzyme activity for a substrate is increased in the presence of another substrate, due to structural or electronic changes of the enzyme, and is often referred to as heterotropic positive cooperativity (activator is different than the substrate). Autoactivation (homotropic positive cooperativity) occurs when a compound activates its own metabolism, as has been observed with such compounds as carbamazepine [3] and

progesterone [4], resulting in a sigmoidal kinetic profile. Kinetic parameters from sigmoidal data are often estimated using the Hill equation, with the Hill coefficient describing the degree of sigmoidicity ($n > 1$). Substrate inhibition occurs when the reaction velocity of product formation decreases once a particular substrate concentration is surpassed, resulting in a convex reaction profile, while partial inhibition is when incomplete inhibition is observed even in the presence of saturating concentrations of inhibitor. The final atypical kinetic phenomenon reported is a biphasic saturation profile, indicative of an enzyme with both a low affinity and a high affinity binding component. This is similar to a dual-enzyme model, but interpreted as a single enzyme with multiple binding regions [3].

Previously, atypical kinetic profiles were most likely overlooked, citing assay or experimental variability, or not observed, perhaps due to lack of sensitive analytical techniques or the paucity of data points collected, particularly at the low end of the substrate concentration range. However, with today's improved analytical technology, scientists in drug metabolism are capable of designing more complete kinetic studies to fully characterize the biotransformation of the compounds of interest, and thus are becoming more cognizant of these atypical kinetic events. This is reflected by the increase in atypical kinetic profiles reported in the literature over the past few years.

1.2. Activation (Heterotropic Positive Cooperativity)

Activation of drug metabolism was first observed many years ago in microsomes from rat tissue, as 7,8-benzoflavone (α -naphthoflavone) was shown to activate the hydroxylation of benzo[a]pyrene in control and phenobarbital treated rats [5]. Several

other studies undertaken shortly thereafter concurred with these findings [6-8], but the particular isoforms involved in this metabolism and activation had not been isolated or identified. Later studies established that purified CYP3A enzymes are directly activated by 7,8-benzoflavone [4;9]. Schwab and colleagues concluded that both progesterone 6 β -hydroxylation and 17 β -estradiol 2-hydroxylation are activated by 7,8-benzoflavone in man and rabbit, based on the observation that the K_m of these reactions decreased and V_{max} increased in the presence of 7,8-benzoflavone. Similarly, Shou and co-workers demonstrated that 7,8-benzoflavone activates phenanthrene metabolism by increasing the V_{max} , although the K_m was unaffected. Also discovered was that 7,8-benzoflavone is a substrate for CYP3A4, as phenanthrene was found to decrease the V_{max} of 7,8-benzoflavone metabolism, without affecting the K_m . This particular finding generated ideas as to the mechanism of activation (discussed later).

Because of the numerous studies showing 7,8-benzoflavone activation of CYP3A4 (Table 1.1), it has been labeled as the classic CYP3A4 activator. However, several groups have observed that compounds other than 7,8-benzoflavone may activate CYP3A4 activity. Maenpaa et al. [10] have shown that testosterone stimulates the formation of 4-hydroxymidazolam from midazolam in liver microsomes and expressed CYP3A4, while Ludwig et al. [11] observed that meloxicam metabolism is activated by quinidine in a dose-dependent manner. Interestingly, quinidine also activates the metabolism of diclofenac to its 5-hydroxy derivative and warfarin to its 4'- and 10-hydroxy metabolite in human liver microsomes, recombinant CYP3A4, and in human hepatocyte suspensions, indicating that this interaction occurs in intact cells, and is likely not an *in vitro* artifact [12;13]. These observations are not altogether surprising,

considering the number of substrates with varying sizes and molecular weights that CYP3A4 accommodates. In fact, Wang et al. [14] suggest that these types of interactions with CYP3A4 are substrate dependent, implying that perhaps the interactions driving these atypical kinetic observations depend at least partially upon a compound's physical and chemical properties. One particularly interesting observation from Wang and colleagues was that 50-100 μ M testosterone inhibited midazolam 1'-hydroxylation, but stimulated the formation of the 4-hydroxylated product, suggesting an alteration of regiospecificity, perhaps due to the presence of testosterone in the active site. In addition, testosterone did not interact at all with nifedipine metabolism, but partially inhibited terfenadine t-butyl-hydroxylation [14].

Just as compounds other than 7,8-benzoflavone can activate CYP3A4, the activity of other isoforms may be stimulated by the presence of certain compounds. For example, Korzekwa et al. [3] have shown that dapsone activates CYP2C9-mediated metabolism of both flurbiprofen and naproxen, albeit to differing degrees, further supporting the contention that these interactions may be substrate dependent. Furthermore, Korzekwa et al. demonstrated that dapsone is also metabolized by CYP2C9, analogous to 7,8-benzoflavone metabolism by CYP3A4 [3]. Thus, there appears to be some possible mechanistic similarities for activation between these isoforms. Table 1.1 shows some of the important examples of activation that have been observed in various expression systems.

1.3. Autoactivation (Homotropic Positive Cooperativity)

With regards to autoactivation, according to a review by Ekins et al. [15], the most frequently reported CYP3A4 substrates that display autoactivation kinetics include aflatoxin B1, amitriptyline, and progesterone, though some other compounds displaying autoactivation kinetics have been reported (Table 1.2). In addition to CYP3A4, it has been demonstrated that *E. coli* expressed and RECO expressed CYP1A2 show autoactivation kinetics for ethoxyresorufin O-deethylation [15]. However, ethoxyresorufin O-deethylation in human liver microsomes and insect expressed sources of CYP1A2 showed classical Michaelis-Menten kinetics, suggesting that atypical kinetic profiles may not only be substrate dependent, but also enzyme source dependent. One other CYP450 that displays autoactivation kinetics is CYP2B6, as Ekins et al. [16] observed that CYP2B6-mediated testosterone 16 β -hydroxylation metabolism data are best fit to the Hill equation ($n=1.3$). More recent evidence suggests that phase II enzymes can display autoactivation kinetics as well. In a study by Fisher et al. [17], kinetic profiles of estradiol-3-glucuronide and acetaminophen-O-glucuronide were best fit to the Hill equation, as Eadie-Hofstee plots of the data were indicative of autoactivation. This observation is the first report involving autoactivation of glucuronosyltransferases in microsomal incubations, although bilirubin conjugation by UGT1A1 in hepatocytes has been shown to display autoactivation kinetics as well [18]. These data suggests that atypical kinetics may not be limited to phase I metabolic reactions.

1.4. Substrate Inhibition

Substrate inhibition is another atypical kinetic phenomenon that has been observed in *in vitro* studies. Although the mechanism of substrate inhibition has yet to be fully determined, it has been described by a two-site model in which one binding site is productive, while the other site is inhibitory and operable at high substrate concentrations, resulting in decreased velocity with increasing concentrations [19]. One early study showed that tenoxicam 5'-hydroxylation by the CYP2C family strongly inhibits its own metabolism at concentrations higher than 100-150 μ M [20]. Interestingly, our lab has demonstrated that piroxicam 5'-hydroxylation also demonstrates substrate inhibition in both human liver microsomes and purified baculovirus expressed CYP2C9, although not in expressed CYP2C9 microsomes (T.S. Tracy et al.-submitted for publication). Yet another CYP2C9 substrate displaying substrate inhibition kinetics is the new COX-2 selective NSAID celecoxib [21]. Furthermore, Spracklin et al. [22] have observed that halothane metabolism by CYP2E1 to trifluoroacetic acid and bromide displays substrate inhibition, one of the few examples of atypical kinetics with CYP2E1. With regard to CYP3A4, Schrag and Wienkers [23] observed that triazolam 1'-hydroxylation displayed substrate inhibition kinetics at higher concentrations, confirmed by an Eadie-Hofstee plot, which showed a hook in the upper quadrant, indicative of substrate inhibition. Most recently, Lin et al. [24] have observed substrate inhibition kinetics for several compounds that are routinely used as probes for particular CYP450 isoforms. For example, CYP1A2-catalyzed ethoxyresorufin metabolism to resorufin, CYP2D6-catalyzed dextromethorphan metabolism to

dextrorphan, as well as testosterone and progesterone metabolism by CYP3A4 all displayed substrate inhibition kinetics [24]. The data for ethoxyresorufin is particularly interesting, considering the work done by Ekins and coworkers [15] suggesting autoactivation of ethoxyresorufin metabolism, not substrate inhibition. At this time, the incongruency of these findings is not readily explained, though as stated earlier, enzyme source and inter-lab variability may be factors. Several examples of substrate inhibition are shown in Table 1.3.

1.5. Partial Inhibition

Partial inhibition occurs when a compound acting as either a substrate or inhibitor for a particular CYP450 isoform incompletely inhibits another substrate's metabolism, even under saturating concentrations of the inhibitor. It has been suggested that both substrate and inhibitor may have access to the reactive oxygen within the active site, forming a substrate-inhibitor-enzyme complex that is still productive [19]. It is no surprise then, that partial inhibition is often observed with CYP3A4, since this enzyme has been shown to accommodate multiple substrates simultaneously. While studying the simultaneous metabolism of testosterone and erythromycin by CYP3A4, Wang et al. [25] observed that the inhibition mechanism for this interaction was not purely competitive. At a fixed concentration of 60 μM testosterone, the rate of 6 β -hydroxylation was greatly reduced by high concentrations of erythromycin, suggesting competitive inhibition between substrates. However, at higher fixed amounts of testosterone (250 and 500 μM), only partial inhibition of 6 β -hydroxylation was observed [25]. This suggested that both compounds were being simultaneously metabolized, and may have distinct binding regions within the active site. Wang and co-workers have also observed partial inhibition

by midazolam and terfenadine of CYP3A4-mediated testosterone metabolism [14]. In a similar study, Korzekwa et al. [3] discovered that phenanthrene displayed partial inhibition of 7,8-benzoflavone metabolism, while not affecting its K_m , suggesting that 7,8-benzoflavone was not displaced from the active site. To date, few other identified examples exist for partial inhibition, although it is likely that this phenomenon has been observed for other CYP isoforms, and not labeled as such, or perhaps saturating inhibitor concentrations were not reached.

1.6. Biphasic Kinetics

A biphasic kinetic profile is characterized by an initial Michaelis-Menten-like increase in velocity with increasing substrate concentration. However, the profile does not become asymptotic, with the reaction profile eventually becoming linear with increasing substrate concentration. This results in the inability to predict an apparent V_{max} and thus, K_m . Some examples include naproxen demethylation by CYP2C9, in which saturation is unobtainable up to 1800 μM , and CYP3A4-mediated naphthalene metabolism to 1-naphthol [3]. More recent evidence for biphasic kinetics includes 7-ethoxycoumarin O-deethylation and aminopyrine N-demethylation in recombinant yeast microsomes expressing rat CYP1A1 and yeast NADPH-P450 reductase [26]. This is the first report showing atypical kinetics of P4501A1-mediated 7-ethoxycoumarin metabolism, an often used probe for CYP1A1 activity. In addition, Oda et al. [27] discovered that levo- α -acetylmethadol (LAAM) demethylation, as well as nor-LAAM demethylation to dinor-LAAM exhibits biphasic Eadie-Hofstee plots in cDNA expressed CYP3A4 enzyme. Most recently, it was discovered that the rates of 4-nitrophenol and formaldehyde production from the O-demethylation of 1-methoxy-4-nitrobenzene by

CYP1A2 isolated from rabbit liver produced biphasic plots [28]. As suggested by the above studies, examples of biphasic kinetics are becoming more prevalent, with several CYP isoforms apparently exhibiting this type of behavior (Table 1.4).

1.7. Mechanistic Models of Atypical Kinetics

Since the first observation of 7,8-benzoflavone activating the metabolism of benzo[a]pyrene, much effort has been put forth to understand the mechanism of this and other atypical kinetic events. Shou et al. [9] first suggested that both substrate and activator may be simultaneously present in the active site, both having access to the reactive oxygen. This conclusion was based on kinetic evidence that 7,8-benzoflavone increased the $V_{\max}$ of phenanthrene metabolism without changing the K_m . Conversely, phenanthrene decreased the $V_{\max}$ of 7,8-benzoflavone metabolism without increasing the K_m , suggesting that neither compound displaces the other from the active site, resulting in near-simultaneous metabolism. This study provided the first indirect evidence that more than one molecule could be present in, and influence the active site. In related and perhaps the most comprehensive initial work examining atypical kinetics, Korzekwa et al. [3] provide further evidence for the theory of multiple binding regions within the active site. This group suggested that a two-site binding model may be used to describe all atypical kinetic observations, including those observed in isoforms other than CYP3A4. Korzekwa and coworkers also demonstrated several of the theoretical saturation profiles for an enzyme showing sigmoidal, biphasic, and substrate inhibition kinetics, comparing the K_m and $V_{\max}$ parameters of the two proposed binding sites. One of the most interesting observations from this study was that of the biphasic metabolism of naproxen by CYP2C9, as well as naphthalene by CYP3A4. The authors suggest that

this profile may also be explained by a two site binding model, one site having a low affinity (K_m), low capacity (V_{max}), the other with high affinity and high capacity. This type of profile is similar to that of a two-enzyme model, except that the data is derived from a single purified or expressed enzyme.

Site-directed mutagenesis studies by Harlow and Halpert [29] have added compelling structural evidence supporting the multi-site hypothesis. Their group constructed a L211F/D214E double mutant of CYP3A4, which was designed to mimic the action of effector (7,8-benzoflavone) by reducing the size of the active site with the bulkier phenylalanine and glutamate residues. Results showed that the double mutant exhibited an increased rate of testosterone and progesterone 6 β -hydroxylation and a lower level of activation by 7,8-benzoflavone, suggesting that the active site was the most likely location of activator (effector) binding. In addition, studies have shown that residues 301, 304, 305, and 309, predicted to be within substrate recognition site 4 of CYP3A4, are also involved in steroid and effector binding [30;31]. Other studies supporting the multi-site hypothesis include those of Ngui et al. [12] and Schrag and Wienkers [23]. Briefly, Ngui and coworkers observed that quinidine stimulated diclofenac metabolism, while maintaining normal access to the ferriheme-oxygen complex, while Schrag and Wienkers observed an alteration of the metabolite ratio (4-hydroxy triazolam/1'-hydroxy triazolam) for triazolam due to the presence of testosterone, referred to as differential kinetics (when an effector activates the metabolism of a given substrate at one position but inhibits metabolism at a different position). In addition, Hosea et al. [32] provide evidence for multiple binding sites for CYP3A4 through spectral binding studies with a number of CYP3A4 substrates and a

series of peptide ligands, while Szklarz and Halpert [33] have generated a three-dimensional homology model of CYP3A4, which shows the ability to dock multiple substrates, or substrate and effector within the active site simultaneously.

In agreement with Korzekwa et al., Lin et al. [24] suggest that substrate inhibition may be explained by a two-site binding model, in which the two sites may either be neighboring or distant from each other within the active site. They hypothesize that in the case of substrate inhibition, one site is favorable for oxidation, while the other site is nonproductive, which differs from the theory that both sites may have access to the reactive oxygen. This is further explained by suggesting that when a substrate binds to the inhibitory site (at high concentrations), the complex (SES) is less capable of forming product than the ES complex. A scheme representing the possible enzyme-substrate-effector interactions and product formation has been presented in a recent paper by Shou et al. [19]. Nonetheless, whether one or the other or both sites are productive will likely depend on the physical and chemical properties of the compounds in question.

Though most studies show evidence for a multi-site binding model, other possibilities such as the presence of an allosteric site have not been completely disproven. Ueng et al. [34] suggested an allosteric model for CYP3A4, although the proximity of the allosteric site relative to the active site is not defined. Another study hypothesized that as many as three conformers of CYP3A4 exist, based on kinetic studies of carbon monoxide binding, depending on the substrate bound to CYP3A4 [35]. This group later suggested that activation of benzo[a]pyrene (BaP) metabolism by 7,8-benzoflavone resulted from an allosteric mechanism in which the presence of 7,8-benzoflavone converts an unproductive enzyme conformation to a productive one, resulting in increased levels of

BaP metabolite [36]. One of the most intriguing explanations for the cause of atypical kinetics is one in which the multiple binding-site model and a conformationally based model are combined, as suggested by Atkins et al. [37]. The author's reasoning is that multiple conformations alone can not account for sigmoidal velocity versus substrate concentration curves, while the multiple binding-site theory does not fully explain the lack of reciprocal effects of certain drug interactions. Therefore, a scheme is proposed by which two enzyme conformers are in equilibrium, and each conformer can bind two substrates at a single active site. Another possibility suggested by Ekins et al. [15] is the contribution of water molecules in the active site and hydrogen bonding. They theorized that the displacement of water molecules by a second substrate or effector may cause fluctuations in the conformation of the enzyme, perhaps leading to changes in active site characteristics and enhanced catalytic activity.

The interaction between CYP450 and cytochrome b5 and oxidoreductase may also be involved in the production of atypical kinetic profiles. It is possible that effector binding increases or enhances the interaction of CYP450 with either or both cytochrome b5 and oxidoreductase. However, Ngui et al. [12] were able to exclude the role of cytochrome b5 in the stimulation of diclofenac metabolism by quinidine, as the effect of quinidine was maximal in the absence of cytochrome b5. In addition, Ngui and coworkers observed no change in cytochrome c reduction by oxidoreductase in the presence of quinidine. Similarly, our lab has observed that flurbiprofen 4'-hydroxylation is activated by dapsone, despite the absence of cytochrome b5 (T.S.T. and J.M.H., unpublished observations). However, in a study using rat liver microsomes by Lee et al. [38], it is suggested that the mechanism of cytochrome P450 activation involves electron

transfer steps, likely from cytochrome b5. This was based on the observation that the formation of NAPQI from acetaminophen was not stimulated by 5 mM caffeine or 50 μ M 7,8-benzoflavone in liver microsomes when supported with cumene hydroperoxide via the peroxide shunt, while approximately 3-fold activation was observed for both caffeine and 7,8-benzoflavone in NADPH-supported incubations [38]. In addition, Lee and coworkers observed that an inhibitory cytochrome b5 antibody significantly diminished the activation of NAPQI formation by caffeine, but not 7,8-benzoflavone, suggesting different mechanisms of activation by these compounds. Interestingly, Yamazaki et al. [39] observed that apo-b5 (without heme), was as effective as holo-b5 (with heme) in stimulating testosterone 6 β -hydroxylation and nifedipine oxidation in a system containing NADPH-P450 reductase, suggesting that b5 may activate CYP3A4 by inducing a conformational change in the enzyme, and not by increasing electron flow. However, it has recently been suggested that apo-b5 may be converted to holo-b5 by a heme transfer reaction from the CYP3A4 preparation, which may explain stimulation of CYP3A4 activity [40].

While much evidence exists for the preceding mechanisms of atypical kinetics, no one theory has been proven conclusively. It is possible that one or a combination of the above- mentioned mechanisms, or some as yet undetermined mechanism(s) is/are likely operable and contributing to the atypical kinetic profiles of many drugs.

1.8. Kinetic Modeling of Atypical Kinetics

Along with observing atypical kinetic profiles comes the task of determining how to properly model the data, so as to generate accurate kinetic parameter estimates. As mentioned previously, atypical kinetic profiles have in the past likely been overlooked or ignored. However, it is now apparent that ignoring or truncating nonhyperbolic data can lead to erroneous kinetic parameter estimates, as standard Michaelis-Menten hyperbolic curves are forced through the data, rather than the use of a more appropriate kinetic model. For example, it has been suggested that substantial underestimation of $V_{\max}$ may occur when substrate inhibition is observed if the high concentration data points are ignored [41]. This is validated by Lin and co-workers, who observed that the estimated $V_{\max}$ values for several reactions showing substrate inhibition were 0.91- to 1.8-fold lower when fitted to the Michaelis-Menten equation, rather than a more appropriate substrate inhibition equation [24]. Likewise, Houston and Kenworthy [41] suggest that for a sigmoidal kinetic profile, either underestimation or overestimation of intrinsic clearance (Cl_{int}) may occur if a hyperbolic curve is forced through the data, with the K_m (S_{50}) term most likely affected. Therefore, it is imperative that the proper kinetic model be used to estimate kinetic parameters from the data, although there may be several solutions that satisfactorily model the same set of data [42]. With respect to the equations used for modeling atypical kinetic data, most have been derived to describe the presence of multiple binding sites within the active site. A comprehensive discussion of the modeling of atypical kinetic data and the equations typically used can be found in several

articles [3;41;43;44], with each showing enzyme-substrate-effector kinetic schemes from which these equations are generated.

1.9. Artifactual Sources of Atypical Kinetics

Though the presence of atypical kinetics appears to be real, it is important to perform experiments in a fashion so as to eliminate any artifactual causes of atypical kinetic profiles. Several examples of artifactual sources of sigmoidicity and substrate inhibition have been mentioned by Houston and Kenworthy [41]. Examples cited include significant substrate depletion, nonspecific binding of substrate to the incubation matrix, cellular active transporter systems, lack of analytical sensitivity, and low substrate solubility. Significant substrate depletion results in deviations from initial rate conditions, and leads to erroneous estimation of kinetic parameters, even in the absence of atypical kinetics. As a result, it is important to monitor substrate consumption, and even effector consumption, if the effector happens to be metabolized as well.

Nonspecific binding to microsomal proteins has been shown to be another potential problem in *in vitro* studies [45;46]. One such example is the observation that addition of 750 $\mu\text{g/ml}$ inactive control microsomes produced a 1.8-fold increase in the apparent S_{50} for amitriptyline metabolism, although no effect on the V_{max} or Hill coefficient was observed [46]. As a result, it may be beneficial to run the experiment at the lowest possible protein concentration to help avoid any artifactual results. With respect to low analytical sensitivity and solubility issues, these cause a lack of confidence in quantitation or “effective” substrate concentration at the low, and high end of the concentration range, respectively. In addition, Houston and Kenworthy suggest attaining

a minimum of ten data points before concluding the presence of atypical kinetic profiles. Meanwhile, Shou et al. [43] suggest attaining upwards of 20-30 data points, especially around the inflection points to better define the fitted curve.

Other considerations when conducting these types of studies may be salt and organic solvent concentrations in the incubation. Schrag and Wienkers [47] have observed that pyrene metabolism by CYP3A4 is sigmoidal in the absence of magnesium, but biphasic in the presence of 10 mM magnesium. In this work, evidence suggested a change in CYP3A4 active site conformation in the presence of magnesium, resulting in the different kinetic profile. In a similar study, Maenpaa et al. [10] observed that the addition of 5 and 50 mM MgCl_2 to microsomal incubations resulted in minimal changes in midazolam 1'-hydroxy and 4-hydroxy metabolites, while the addition of 150 mM MgCl_2 resulted in substantial inhibition of both metabolites. Meanwhile, 1'-hydroxy and 4-hydroxy midazolam formation was completely inhibited by 30 mM CaCl_2 in microsomal incubations. In another study, acetonitrile and acetone were found to activate the NADPH-dependent tolbutamide hydroxylation nearly 3-fold in human liver microsomes and CYP2C9-reconstituted system when incubated at 2 to 4% final organic solvent concentrations [48]. From these examples, it is evident that investigators must remain cognizant of the many different conditions that may be artifactual sources of atypical kinetics. In addition, the above list is probably not all inclusive and thus, there may be other confounding factors involved in the observation of atypical kinetics as well.

1.10. *In vivo* Considerations

Although it is known that atypical kinetics exist *in vitro*, an important question is whether they also occur *in vivo*, since they may have considerable pharmacological or

toxicological implications. However, according to Houston and Kenworthy [41], whether or not these types of interactions occur *in vivo*, and if they are clinically relevant is not the primary issue. Even if these interactions are not observed *in vivo*, their presence *in vitro* will affect how data is scaled from the *in vitro* to the *in vivo* situation. Thus, it is best to fully describe the *in vitro* data and then abstract the useful parameter(s) for extrapolation [41]. Nonetheless, *in vivo* studies designed to determine the presence of atypical kinetics would be beneficial, but only a few have actually been performed. The first example of activation of drug metabolism *in vivo* were studies by Lasker et al. [49;50], who observed that the i.p. administration of flavone, nobiletin, tangeretin or 7,8-benzoflavone concurrently with zoxazolamine immediately stimulated the total body metabolism of zoxazolamine to its 6-hydroxy metabolite in neonatal rats in a time course consistent with activation. This activation was found to be dose and dosing-time dependent, as little activation of zoxazolamine metabolism was observed with a low dose of zoxazolamine, as well as when the activator was administered 2-4 hours before zoxazolamine [50]. It is noteworthy that Lasker and co-workers explored the possibility that the flavone compounds could activate zoxazolamine metabolism by displacing it from binding sites on plasma protein or from binding sites in the total body homogenates by performing equilibrium dialysis studies. Results showed no difference in zoxazolamine binding in the presence of flavone. Based on this observation, it was determined that this activation in rats was likely CYP450-mediated, and thus the first noted example of *in vivo* activation of drug metabolism.

More recently, it has been discovered that activation of CYP450-mediated drug metabolism may be observed *in vivo* in monkeys. In previous studies, Tang et al. [51]

observed that the formation of diclofenac metabolites via CYP3A4 increased more than 4-fold in human liver microsomes in the presence of quinidine. Since similar results were seen in monkey liver microsomes, Tang and co-workers followed this work with an *in vivo* study in monkeys and observed that in the presence of quinidine, diclofenac clearance was enhanced in three male rhesus monkeys by 57, 56, and 56%, respectively [51]. The plasma protein binding and blood/plasma ratio of diclofenac remained unchanged in the presence of quinidine, and plasma samples were taken in a time frame too short for the de novo synthesis of CYP proteins. This suggested that the interaction was CYP450-mediated and the reduction in diclofenac levels compared to control was not due to induction [51]. To date, no *in vivo* studies have been conducted to see if this atypical kinetic phenomenon may be observed in humans.

1.11. Cytochrome P450 2C9

With the substantial increase in examples of atypical kinetics over the past few years, much work needs to be done to better understand and predict these events and the contributing isoforms. To date, most work has been done studying atypical kinetics in CYP3A4. However, as mentioned in the previous sections, other CYP450 isoforms have been shown to exhibit atypical kinetics as well, including the CYP2C9 isoform. Few studies have been conducted on the atypical kinetics displayed by this isoform. As a result, the work described herein will focus on the CYP2C9 isoform, and factors that regulate its activity, including exploring potential mechanisms of atypical kinetics, and whether they occur *in vivo*.

The CYP2C enzyme subfamily found in the human liver comprises approximately 20% of total hepatic CYP enzymes [52;53], with CYP2C9 being the major isoform. The CYP2C9 protein is composed of 490 amino acids, with a calculated molecular mass of ~55.6 KDa, and is responsible for metabolizing a vast array of xenobiotic substrates, most of which are weak acids with pKa values ranging from 3.8-8.1 [54]. Examples include (S)-warfarin [55], phenytoin and tolbutamide [56], and several non-steroidal anti-inflammatory drugs, including diclofenac [57], ibuprofen [58], piroxicam [20], naproxen [59;60], and flurbiprofen [61;62]. A comprehensive review of CYP2C9 substrates and inhibitors has been presented by Miners and Birkett [54]. Interestingly, CYP2C9 is polymorphic, with three alleles originally being identified as wild-type (CYP2C9*1), the R144C variant (CYP2C9*2), and the I359L variant (CYP2C9*3), and allele frequencies of 0.86, 0.08 and 0.06, respectively [63;64]. Recently, however, an I359T (CYP2C9*4) allelic variant was identified in Japanese epileptic patients [65], while a D360E (CYP2C9*5) variant was discovered that is apparently only expressed among African Americans at an allele frequency of 0.03 [66]. These allelic variants of CYP2C9 have been shown to variably affect the metabolism of a number of CYP2C9 substrates. For example, CYP2C9*2 (R144C) allelic variant has been shown to reduce the $V_{\max}$ for tolbutamide methyl-hydroxylation by up to threefold [64;67]. In addition, Crespi and Miller [68] observed that the R144C variant exhibited similar apparent K_m values for (S)-warfarin, diclofenac, and lauric acid hydroxylation compared to wild-type CYP2C9 enzyme, but had slower rates of metabolism. Meanwhile, CYP2C9*3 has a reduced capacity to metabolize (S)-warfarin [69], while CYP2C9*2 seems to not affect the apparent K_m for (S)-warfarin 7-hydroxylation [67]. Lastly, the CYP2C9*4 variant has

been shown to decrease the apparent intrinsic clearance of diclofenac compared to wild-type, primarily due to an effect on $V_{\max}$ [70]. Although the allele frequencies for these variants are small, the fact that these amino acid changes generally cause a decrease in metabolic capacity has led to many questions about enzyme active site structure, and recognition of substrates.

It has been proposed that CYP2C9, along with other CYP2 family isoforms, has six substrate recognition sites (SRSs), which variably contribute to the recognition and binding of certain substrates [71]. Many studies have evolved from this finding in an attempt to better understand the substrate selectivity of CYP2C9, as well as the binding interactions within the active site and overall three-dimensional structure of this protein. Initially, a three-dimensional model of CYP2C9 was constructed by Korzekwa and Jones [72], which was based on amino acid sequence alignment data and the crystal structure of the bacterial enzyme CYP101. This homology model was considered to have low resolution, and thus of limited utility in the prediction of substrate binding by CYP2C9. Other molecular modeling techniques, such as pharmacophore modeling, have been performed to probe the active site binding interactions of CYP2C9. Jones et al. [73] rationalized the substrate specificity of nine substrates and one inhibitor of CYP2C9 in terms of a hydrogen bond donor/acceptor model to propose an active site template model. This model was described by A, the distance between the site of metabolism (Y) and the hydrogen bond donor heteroatom (X), and C, the angle between this and the hydrogen bond, with mean dimensions for the nine substrates and one inhibitor of $A = 6.7 \pm 1.0 \text{ \AA}$, $C = 133 \pm 21^\circ$. Mancy et al. [74] generated a pharmacophore model by analyzing the ability of 10 derivatives of tienilic acid, a known substrate of CYP2C9, to be

metabolized. Results showed that only derivatives containing an acidic function underwent a 5-hydroxylation of their thiophene ring like tienilic acid. In addition, the nonacidic derivatives were unable to competitively inhibit the hydroxylation of tienilic acid, suggesting that they not only did not undergo hydroxylation, but also were not recognized by the CYP2C9 enzyme. This suggested that most substrates for CYP2C9 interact through their acidic anionic site with a cationic residue of the CYP2C9 protein. One study in particular observed that arginine 97 is important for diclofenac hydroxylation, suggesting the presence of an important cationic binding region in the active site, since when this amino acid was mutated to a neutral moiety, diclofenac hydroxylation was greatly reduced [75]. Other studies confirm the importance of an anionic binding region within the CYP2C9 active site [76;77], both of which also mention the importance of a hydrophobic zone between the substrate hydroxylation site and the anionic site. In addition, a molecular model of CYP2C9 was generated using the 3D-QSAR method CoMFA (comparative molecular field analysis) and 29 coumarin ring-containing compounds to evaluate both steric and electrostatic interactions within the active site[78]. This predictive model has since been updated with 14 new compounds [79], due in part to the findings of Haining et al. [80] who observed the importance of a hydrophobic residue within the CYP2C9 active site. More specifically, it was observed that an F114L mutant decreased the $V_{\max}$ for (S)-warfarin 7-hydroxylation 4-fold, and increased the K_m 13-fold, suggesting the ability of certain aromatic ligands to π -stack to the F114 residue, with the L114 residue apparently eliminating this interaction. Another QSAR study analyzing CYP2C9 competitive inhibitors has been performed by Ekins et al. [81]. This group generated a 3D/4D QSAR pharmacophore model of the common

structural features of CYP2C9 inhibitors using Catalyst, a commercially available 3D-QSAR technique that does not require manual alignment of molecules, unlike the CoMFA studies of CYP2C9 [78;79]. Meanwhile, Afzelius et al. [82] generated a 3D-QSAR model for 29 structurally diverse competitive inhibitors of CYP2C9, and performed molecular modeling studies with the help of a homology model of CYP2C9, based on the coordinates of rabbit CYP2C5, the first mammalian CYP enzyme for which there is a crystal structure [83]. Results from this study showed the ability to predict K_i values to within 0.5 log units of the experimental K_i values, which was more robust than the QSAR method by Ekins et al. [81].

Cytochrome P450C9 has usually been considered to be a well-behaved enzyme that follows the rules of normal Michaelis-Menten type kinetics. However, it is now becoming clear that CYP2C9 shows some atypical kinetic profiles similar to CYP3A4, most notably activation by dapsone [3]. In this study, Korzekwa's group suggested that activation, as well as other atypical kinetics events, may be explained by a model that suggests the presence of multiple binding regions within the CYP450 active site, each having its own kinetic characteristics. However, only one group has explored this theory in their pharmacophore models. This study explored the differences in metabolic stability between warfarin and phenprocoumon by CYP2C9, and provided evidence to support the multiple binding theory, as two binding sites were proposed, a π -stacking site and an ionic binding site for organic anions [84]. This finding is similar to the other pharmacophore studies previously mentioned, but is the first to interpret these sites as being distinct regions in the active site. Despite these suggestions, minimal kinetic data from CYP2C9 has been generated to provide evidence for this theory. The studies

described herein show the ability to kinetically model activation of CYP2C9-mediated metabolism of several compounds by dapsone with a two-binding site effector model, along with further evidence for the presence of multiple binding regions within the CYP2C9 active site. In addition, other factors involved in the mechanism of activation of CYP2C9 will be explored to provide information necessary for eventually generating a pharmacophore model of multiple binding regions within the CYP2C9 active site.

1.12. Research Study Objectives

1. Kinetically model dapsone activation of CYP2C9-mediated flurbiprofen 4'-hydroxylation, naproxen O-demethylation, and piroxicam 5'-hydroxylation, as well as explore the effect of flurbiprofen on dapsone N-hydroxylation.
2. Determine the structural requirements of dapsone activation of CYP2C9 by screening 17 analogs of dapsone for activation of flurbiprofen, naproxen, and piroxicam metabolism in human liver microsomes, followed by full kinetic studies to observe the effect of different concentrations of effector on CYP2C9 activity.
3. Explore other studies for understanding the mechanism of activation of CYP2C9, including the use of CYP2C9/19 chimeric enzymes, CYP2C19 mutant enzymes, spectral binding studies with CYP2C9, activation of CYP2C9 allelic variants, dapsone metabolite activation of CYP2C9, and alteration of regiospecificity of ibuprofen metabolism.

4. Validate a urine and plasma HPLC assay for use in analyzing samples to determine if activation of flurbiprofen 4'-hydroxylation by dapsone also occurs *in vivo* in humans.
5. Analyze urine and plasma samples and perform pharmacokinetic modeling for a clinical study exploring the ability of dapsone to activate flurbiprofen 4'-hydroxylation in 12 human subjects.

Table 1.1. Reported Examples of Activation Kinetics

Examples of activation kinetics (heterotropic positive cooperativity) reported in the literature in various *in vitro* systems.

Enzyme Source	Substrate	Pathway	Effector	Reference
RLM ^a	Benzo[a]pyrene	Hydroxylation	7,8-benzoflavone	[5]
HLM ^b	Benzo[a]pyrene	Hydroxylation	7,8-benzoflavone	[7]
HLM	Progesterone	6 β -hydroxylation	7,8-benzoflavone	[4]
HLM	17 β -estradiol	2-hydroxylation	7,8-benzoflavone	Ibid.
HepG2 expressed CYP3A4	Phenanthrene	Hydroxylation	7,8-benzoflavone	[9]
HepG2 expressed CYP3A4	Phenanthrene	Hydroxylation	7,8-benzoflavone	[3]
E. coli purified CYP3A4	AflatoxinB1	8,9-epoxidation	7,8-benzoflavone	[34]
E. coli purified CYP3A4	Carbamazepine	10,11-epoxidation	7,8-benzoflavone	Ibid.
HLM	Diazepam	Hydroxylation	7,8-benzoflavone	[85]
HLM	Diazepam	N-demethylation	7,8-benzoflavone	Ibid.
HLM	Phenacetin	O-deethylation	7,8-benzoflavone	[86]
HLM	Midazolam	4-hydroxylation	Testosterone	[10]
HLM and B-lymphoblastoid expressed CYP3A4 ^c	Midazolam	4-hydroxylation	Testosterone	[14]
HLM and B-lymphoblastoid expressed CYP3A4 ^c	Meloxicam	5'-methylhydroxylation	Quinidine	[11]
HLM and B-lymphoblastoid expressed CYP3A4 ^c	Diclofenac	5-hydroxylation	Quinidine	[12]
HLM and B-lymphoblastoid expressed CYP3A4 ^c	Warfarin	4'-and 10-hydroxylation	Quinidine	[13]
Baculovirus purified CYP2C9	Flurbiprofen	4'-hydroxylation	Dapsone	[3]

^a rat liver microsomes, ^b human liver microsomes, ^c obtained from Gentest

Table 1.2. Reported Examples of Autoactivation Kinetics

Examples of autoactivation kinetics (homotropic positive cooperativity).
For a more comprehensive review, see Ekins et al., 1998a.

Enzyme Source	Substrate	Pathway	Reference
Baculovirus expressed CYP3A4	Diazepam	Hydroxylation	[43]
Baculovirus expressed CYP3A4	Diazepam	Demethylation	Ibid.
Vaccinia expressed CYP2C9	Dapsone	Hydroxylation	[3]
B-lymphoblastoid expressed CYP2B6 ^a	Testosterone	16 β -hydroxylation	[16]
HLM ^b	Estradiol	3-glucuronidation	[17]
HLM	Acetaminophen	O-glucuronidation	Ibid.

^a obtained from Gentest, ^b human liver microsomes

Table 1.3. Reported Examples of Substrate Inhibition Kinetics

Substrates reported to display substrate inhibition kinetics in various *in vitro* systems.

Enzyme Source	Substrate	Pathway	Reference
HLM ^a	Tenoxicam	5'-hydroxylation	[20]
cDNA-expressed CYP2E1 ^b	Halothane	TFA formation ^c	[22]
cDNA-expressed CYP2E1 ^b	Halothane	Bromide formation	Ibid.
HLM	Fluoxetine	Demethylation	[87]
HLM	Fluoxetine	Demethylation	[88]
HLM	Triazolam	1'-hydroxylation	[23]
HLM	Celecoxib	Hydroxylation	[21]
Baculovirus expressed CYP2C9	Celecoxib	Hydroxylation	[24]
Baculovirus expressed CYP1A2	Ethoxyresorufin	Resorufin formation	Ibid.
Baculovirus expressed CYP3A4	Benzyloxyresorufin	Resorufin formation	Ibid.
Baculovirus expressed CYP2D6	Dextromethorphan	O-demethylation	Ibid.
Baculovirus expressed CYP3A4	Progesterone	6 β -hydroxylation	Ibid.
Baculovirus expressed CYP3A4	Testosterone	6 β -hydroxylation	Ibid.

^a human liver microsomes, ^b obtained from Gentest, ^c trifluoroacetic acid

Table 1.4. Reported Examples of Biphasic Kinetics

Substrates reported to display biphasic kinetics in various *in vitro* systems.

Enzyme Source	Substrate	Pathway	Reference
Vaccinia expressed CYP2C9	Naproxen	O-demethylation	[3]
Baculovirus expressed CYP3A4	Naphthalene	Hydroxylation	Ibid
Yeast expressed CYP1A1	7-ethoxycoumarin	O-deethylation	[26]
Yeast expressed CYP1A1	Aminopyrine	N-demethylation	Ibid.
HLM ^a and cDNA-expressed CYP3A4	Levo- α -acetylmethadol	Demethylation	[27]
HLM and cDNA-expressed CYP3A4	Nor-levo- α -acetylmethadol	Demethylation	Ibid.
<i>E. coli</i> purified CYP1A2 ^b	1-methoxy-4-nitrobenzene	O-demethylation	[28]

^a human liver microsomes, ^b rabbit enzyme

CHAPTER II

Dapsone Activation of CYP2C9-Mediated Metabolism: Evidence for Activation of Multiple Substrates and a Two-Site Model

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2.1. Introduction

It is becoming evident that many cytochrome P450-catalyzed reactions do not proceed with typical Michaelis-Menten kinetics. Observations of atypical kinetic events such as activation, autoactivation, and substrate inhibition are becoming increasingly common, with the term “cooperativity” often being used to describe these types of phenomena. To date, CYP3A4 has been the most studied isoform with respect to atypical kinetics due to the large number of reactions that it catalyzes and the complexity of its active site. However, other relevant P450s have been shown to participate in a number of these abnormal kinetic events [1-3], making analysis of *in vitro* data problematic. As a result, it has been suggested that atypical kinetics must be considered during *in vitro-in vivo* scaling of kinetic data. Houston and Kenworthy [4] have demonstrated that failure to properly assess atypical kinetics may result in under or overestimation of *in vivo* clearance of drugs when based on *in vitro* kinetic parameter estimates. Therefore, it is imperative that the cause and mechanism of these events be determined, as well as the isoforms affected.

Our lab has previously demonstrated that CYP2C9-mediated flurbiprofen 4'-hydroxylation and naproxen demethylation are activated by dapsone in human liver microsomes and baculovirus-expressed CYP2C9 [2]. However, kinetic effects of this activation have yet to be fully characterized. While flurbiprofen is exclusively metabolized by CYP2C9 [5] and shows normal Michaelis-Menten kinetics [6], naproxen is metabolized by CYP2C9 and CYP1A2 [7;8] and has been shown to exhibit non-saturable biphasic kinetics in microsomes [7], as well as in expressed CYP2C9 [2]. The unsaturable metabolic profile observed in expressed CYP2C9 suggests that there may be two binding components within the CYP2C9 active site, one with a low K_m , low V_{max} , the other with a high K_m and

high $V_{\max}$, similar to naphthalene metabolism by CYP3A4 [2]. Piroxicam, another NSAID reported to be metabolized by CYP2C9 [9], has been shown to exhibit atypical kinetics as well (substrate inhibition) in baculovirus expressed CYP2C9 (unpublished results), and has a quite different structure than either flurbiprofen or naproxen.

Dapsone is a compound used to treat *Pneumocystis carinii* pneumonia in AIDS patients, and is metabolized by CYP2C9 at therapeutic concentrations to its hydroxylamine metabolite [10;11]. Thus, it appears that dapsone is also a substrate for the enzyme it activates, suggesting that dapsone may cause activation by binding in the active site of CYP2C9. This scenario is analogous to 7,8-benzoflavone, which activates CYP3A4-mediated phenanthrene metabolism, in addition to being a substrate for CYP3A4 [1].

The purpose of the current study was to determine the effect of dapsone on the kinetics of (S)-flurbiprofen, (S)-naproxen, and piroxicam, three prototypical substrates of CYP2C9 with differing structures and kinetic profiles (Figure 2.1). It was hypothesized that a kinetic model which suggests that both the substrate and effector may be present in the active site simultaneously, both having access to the active oxygen was applicable [1;2;12]. Thus, kinetic data from expressed CYP2C9 were fit to the two-site model (equation 2.3) in addition to either the Michaelis-Menten equation (equation 2.1) or a two-site biphasic model (equation 2.2). If our hypothesized two-site model for CYP2C9 is accurate, and the activator (dapsone) also serves as a substrate, then theoretically, near-simultaneous metabolism should occur, and metabolites from both compounds should be measurable. Therefore, the effect of flurbiprofen on dapsone hydroxylation kinetics was also examined by measuring dapsone hydroxylamine formation and fitting data to the Michaelis-Menten equation.

2.2. Methods

Chemicals and reagents. Acetonitrile and dibasic potassium phosphate were obtained from Fisher Scientific Co. (Pittsburgh, PA). (S)-Flurbiprofen, 4'-hydroxy flurbiprofen, and 2-fluoro-4-biphenyl acetic acid (internal standard) were gifts from Pharmacia, Inc. (Kalamazoo, MI). (S)-naproxen and desmethylnaproxen were gifts from Syntex Laboratories Inc. (Palo Alto, CA). Piroxicam and dapsone were obtained from Sigma Chemical Co. (St. Louis, MO), while 5'-hydroxy piroxicam was a gift from Pfizer Inc. (Groton, CT.). Dapsone hydroxylamine was synthesized by the method of Uetrecht et al. [13] and was a gift from Robert Branch at the University of Pittsburgh. All other chemicals were obtained from commercial sources and were of the highest purity available.

Incubation Conditions. Incubation mixtures in a 6X6 matrix design contained 1 pmol purified CYP2C9 (5 pmol for piroxicam) co-expressed with reductase (Camitro Corporation, Menlo Park, CA), with either (S)-flurbiprofen (2-300 μ M), (S)-naproxen (10-1800 μ M), or piroxicam (5-900 μ M) incubated with dapsone (0-100 μ M) in 50 mM potassium phosphate buffer at pH 7.4. Final incubation volume was 0.2 ml and reactions were initiated with 1 mM NADPH and allowed to incubate at 37°C for 20 minutes (45 minutes for piroxicam). Incubations with flurbiprofen were quenched by adding 200 μ l acetonitrile containing internal standard (180 ng/mL 2-fluoro-4-biphenylacetic acid), followed by addition of 40 μ l 1/2-strength H_3PO_4 . Incubations with naproxen were quenched by adding 200 μ l acetonitrile followed by addition of 40 μ l 1/2-strength H_3PO_4 , while piroxicam reactions were quenched by adding 20 μ l perchloric acid, followed by directly placing incubation tubes on ice. Samples from all incubations were then centrifuged at 10,000 rpm for 5 minutes, placed into autosampler vials,

and 10-100 μ l injected onto HPLC system. In experiments examining flurbiprofen effect on dapsone hydroxylamine formation, flurbiprofen concentrations were 0, 2, 5, and 10 μ M, with dapsone concentrations ranging from 1-300 μ M. To prevent degradation of the dapsone hydroxylamine metabolite, 1 mM ascorbic acid was included in the 50 mM potassium phosphate buffer in those cases where this metabolite was being measured. The reaction was stopped by the addition of 0.1 ml of cold methanol. Samples were then spun at 20,000g for 5 minutes at 3°C in an Eppendorf 5417R centrifuge and 15 μ l injected onto an LC/MS system for immediate dapsone hydroxylamine analysis to prevent instability issues. All other conditions were as described above.

Analysis of 4'-hydroxy flurbiprofen. 4'-Hydroxy flurbiprofen was analyzed by HPLC with fluorescence detection and comparison to a standard dissolved in 50:50 (v/v), acetonitrile:50 mM potassium phosphate buffer pH 7.4. The HPLC system consisted of a Waters® 501 HPLC pump, a Waters® 717 autosampler and a Waters® 470 fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm. The mobile phase consisted of 45:55 (v/v), acetonitrile:20 mM K₂HPO₄, pH 3.0 pumped at 1 ml/min through a Brownlee Spheri-5® C₁₈ 4.6 x 100 mm column. 4'-Hydroxy flurbiprofen and internal standard eluted at approximately 2.2 and 4.8 minutes, respectively.

Analysis of desmethylnaproxen. Desmethylnaproxen was analyzed identically to 4'-hydroxy flurbiprofen except that the fluorescence detector was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm, and no internal standard was used. Desmethylnaproxen eluted at approximately 2.1 minutes.

Analysis of 5'-hydroxy piroxicam. 5'-Hydroxy piroxicam was analyzed by HPLC with UV detection (365 nm). The HPLC system was as described above, but was fitted

to a Waters[®] 486 UV detector. The mobile phase consisted of 50:50 (v/v), acetonitrile:50 mM K₂HPO₄, pH 3.0 pumped at 1 mL/min through a Phenomenex Luna C₁₈ 5 µm, 4.6 x 150 mm column. Retention time for 5'-hydroxy piroxicam was approximately 4.3 minutes.

Analysis of dapsone hydroxylamine. HPLC/ESI-MS was performed on a Perkin Elmer Sciex API 150ex single quadrupole mass spectrometer connected to a Perkin Elmer Series 200 LC pump and autosampler. The mass spectrometer was fitted with a turbo ion-spray source and operated in positive-ion SIM (selective ion monitoring) electrospray mode, with an ESI spray voltage of 5.0 kV and source temperature of 450°C. The turbo ion-spray gas flow rate was set at 7000 cc/min. Tuning of the ESI source and MS was accomplished by infusing a solution containing 20 µg/ml dapsone hydroxylamine into the mobile phase flow path post column via a T connector using a Harvard Apparatus (Holliston, MA) manual injector. The instrument was controlled by a Dell Optiplex GX1 computer running Analyst version 1.1 software. Dapsone hydroxylamine formation was confirmed by appearance of the MH⁺ ion at *m/z* of 265.1, and chromatographic retention time corresponding to that of a synthetic standard dissolved in DMSO. Stability of dapsone hydroxylamine metabolite was exhibited over the time required to analyze all of the incubation samples. Analytical separation was accomplished using a Zorbax SB-C₈ 2.1 x 50 mm, 5 µm microbore column with a gradient elution profile. Solvent flow through the column was 0.7 ml/min with 30% of the flow being diverted to the mass spectrometer. Gradient elution consisted of mobile phase A [Methanol] and mobile phase B [100 mM Ammonium Acetate (pH 4.38)]. The gradient was initiated at a ratio of 10%A:90%B. This ratio was altered in a linear fashion to 80%A:20%B over 1 minute, and held for 2.4 minutes to allow elution of dapsone hydroxylamine. The system was then returned to initial conditions

over 0.1 minutes and allowed to equilibrate for 4.4 minutes prior to the next injection. The flow was diverted to waste for 1 minute post injection to prevent fouling of the interface from polar compounds in the solvent front. Dapsone hydroxylamine eluted at approximately 2.9 minutes.

Data Analysis and Equations. Kinetic parameters for flurbiprofen, piroxicam, and dapsone hydroxylation were estimated using the nonlinear regression function in Sigma Plot (Version 6.0), and the standard Michaelis-Menten velocity equation:

$$v = \frac{V_m \bullet S}{K_m + S} \quad (2.1)$$

Kinetic parameters for naproxen demethylation were estimated using a two-site biphasic equation:

$$v = \frac{(V_{m1} \bullet S) + Cl_{int} \bullet S^2}{(K_{m1} + S)} \quad (2.2)$$

where Cl_{int} is referred to as intrinsic clearance and represents the linear portion of the biphasic kinetic curve (V_{max2}/K_{m2}).

The same data (36 points) from fits to equations 2.1 and 2.2 were also fit to a two-site model equation [2] utilized for surface plot analysis of flurbiprofen 4'-hydroxylation, naproxen demethylation and piroxicam 5'-hydroxylation in the presence of dapsone was as follows:

$$v = \frac{V_{max} \bullet [S]}{K_m \left(\frac{1 + \frac{[B]}{K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right) + [S] \left(\frac{1 + \frac{[B]}{\alpha K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right)} \quad (2.3)$$

where S is the substrate, B is the effector, V_{max} and K_m are kinetic constants for substrate metabolism, K_B is the binding constant for the effector, α is the change in K_m resulting from effector binding, and β is the change in V_{max} from effector binding. For activation, $\alpha < 1$ and/or $\beta > 1$.

2.3. Results

Dapsone Activation of Purified CYP2C9. The kinetic parameters for flurbiprofen and piroxicam hydroxylation were determined by fitting data to the Michaelis-Menten equation (equation 2.1), while parameters for naproxen demethylation were estimated by fitting data to a two-site biphasic model equation (equation 2.2). Dapsone activated flurbiprofen 4'-hydroxylation in purified CYP2C9 (Figure 2.2), increasing $V_{\max}$ from 12.6 to 20.6 pmol/min/pmol P450 while lowering the K_m from 28.9 to 10.0 μM (Table 2.1). Meanwhile, the Figure 2.3 shows that the $V_{\max}$ for piroxicam hydroxylation was increased from 0.08 to 0.20 pmol/min/pmol P450, with the K_m reduced from 183 to about 50 μM (Table 2.1). Likewise, naproxen demethylation was activated by the presence of dapsone with the data fit to a biphasic equation reflecting the kinetic profile of the reaction (Figure 2.4). The $V_{\max}$ for naproxen demethylation was increased from 11.7 to 34.7 pmol/min/pmol P450, while the K_m was decreased from 433 to 67.4 μM (Table 2.2), a greater-fold change than observed with either flurbiprofen or piroxicam. While the Clint ($V_{\max 2}/K_{m 2}$) value did not change, it contributed to a lesser extent in defining the kinetic profile of naproxen in the presence of dapsone (Table 2.2). This is observed by the increase in Clint of the first naproxen binding site ($V_{\max 1}/K_{m 1}$) from 0.027 with no dapsone to 0.52 in the presence of 100 μM dapsone, thus displaying that the Clint of the second site ($V_{\max 2}/K_{m 2}$) contributes a smaller percentage to total naproxen demethylation when dapsone is present. Kinetic parameters for flurbiprofen, naproxen and piroxicam metabolism were also estimated using the two-site model equation (equation 2.3). Data from Table 2.3. further suggests that dapsone activates the metabolism of these three compounds, as all α values are below 1, and all β values are above 1. It is also

interesting to note that the binding constant for dapsone varies according to the substrate being activated (Table 2.3). The three-dimensional activation plot for flurbiprofen can be seen in Figure 2.5, demonstrating activation of flurbiprofen hydroxylation by dapsone. However, flurbiprofen appeared to not substantially affect the formation of dapsone hydroxylamine, as similar velocity curves were obtained in the presence of 0, 2, 5, and 10 μM flurbiprofen (Figure 2.6). V_{max} values for dapsone hydroxylation ranged from 36-44 pmol/min/pmol P450, while K_m values ranged from 81-144 μM (data not shown). Similarly, a three-dimensional plot exhibiting activation of naproxen demethylation is shown in Figure 2.7, while in Figure 2.8, an Eadie-Hofstee plot shows the effect of dapsone on the kinetic profile of naproxen. In the absence of dapsone, a biphasic plot is observed, whereas the presence of 100 μM dapsone causes the biphasic profile of naproxen to become linear. As a result, naproxen demethylation in the presence of 100 μM dapsone was also fit to the Michaelis-Menten curve, with a V_{max} of 37.0 pmol/min/pmol P450 and a K_m of 80 μM (Table 2.2). Lastly, a three-dimensional plot in Figure 2.9 shows that piroxicam, a structurally different CYP2C9 substrate, is also activated by dapsone.

Stability of Dapsone Hydroxylamine. Dapsone hydroxylamine is known to be an unstable compound in potassium phosphate buffer [14;15]. A standard solution was prepared in potassium phosphate buffer containing 1 mM ascorbic acid and a 15 μl injection was made from the same vial every 20 minutes at room temperature and analyzed according to methods described in Materials and Methods. When subjected to our incubation conditions over 5 hours, the peak area coefficient of variation was 2.3%

(data not shown), demonstrating that quantitation would not be affected by metabolite instability.

2.4. Discussion

Results from this study suggest that dapsone activates multiple substrates of CYP2C9, being described by a model in which dapsone (effector) and substrate may fit in the active site simultaneously. The increase in $V_{\max}$ and decrease in K_m for flurbiprofen 4'-hydroxylation while dapsone hydroxylamine metabolite is also measurable provide additional evidence for this theory. In addition, the naproxen metabolic profile was changed from biphasic to hyperbolic in the presence of dapsone, and piroxicam, a third CYP2C9 substrate with a distinct structure, was also metabolically activated by dapsone.

It was once believed that most P450-mediated reactions obeyed normal Michaelis-Menten enzyme kinetics, allowing for relatively simple prediction of kinetic parameters. However, atypical CYP450 kinetics are now being recognized more frequently in the area of drug metabolism, which complicates scaling kinetic data from the *in vitro* to the *in vivo* situation. To date, CYP3A4 has been the most studied isoform in terms of atypical kinetic behavior. For example, studies by Schwab et al. [16] and Shou et al. [1] have shown that purified CYP3A4 enzymes are directly activated by 7,8-benzoflavone. This is particularly interesting because like dapsone, 7,8-benzoflavone is a substrate for the enzyme that it activates. More recent data suggests that P450 enzymes other than CYP3A4 may behave in an atypical manner. Korzekwa et al. [2] suggest that CYP1A1 mediated naphthalene metabolism shows a sigmoidal saturation curve and that

Hep G2 expressed CYP2B6, CYP2C8, CYP2C9, and CYP3A5 may also exhibit non Michaelis-Menten kinetics, depending on the substrate. Likewise, Ekins et al. [3] have shown that CYP1A2 displays autoactivation kinetics for ethoxyresorufin metabolism, although results appear to be enzyme source dependent. Despite these studies describing activation, no clear mechanism has been determined, although many models have been proposed. Ueng et al. [17] have described a model which suggests that an allosteric site is involved in the cooperativity of certain CYP3A4 reactions, but the proximity of this site is not defined. Harlow and Halpert suggest that effector binding is most likely in the active site along with the substrate, but go on to propose that oxidation only occurs at one binding region within the active site [18]. Another possibility that has been put forth is the idea of kinetically distinguishable conformers of CYP3A4 [19], which was proposed based on kinetic studies of carbon monoxide binding. However, the model we believe that most accurately describes the activation phenomenon is that of Shou and Korzekwa [1;2;12], which suggests that both substrate and effector are bound in the active site simultaneously with distinctive binding regions and access to the reactive oxygen. It should be stated that this model does not distinguish between allosteric or active site effector binding.

From our experiments, we have observed near-simultaneous metabolism of flurbiprofen and dapsone, although it is likely that there is only one productive orientation at a given time. Nonetheless, this provides support to the primary hypothesis that substrate and activator may bind in the active site simultaneously, potentially ruling out the theory of an allosteric site causing cooperativity. The main basis for this hypothesis is that dapsone is also a substrate for CYP2C9. Although the CYP2C9 active

site is not as large as CYP3A4, it still may be able to accommodate two rather small compounds. In fact, NMR studies by Banci et al. [20] have shown that both pyridine and imidazole can fit into the P450cam active site simultaneously. Furthermore, flurbiprofen 4'-hydroxylation, naproxen demethylation, and piroxicam 5'-hydroxylation K_m values were all decreased in the presence of dapsone (Tables 2.1 and 2.2), suggesting that dapsone does not displace these compounds from the active site. An interesting discovery which provides further evidence to the two-site hypothesis is the kinetic profile of naproxen. In the absence of dapsone, naproxen exhibits a biphasic kinetic profile, as shown by the Eadie-Hofstee plot in Figure 2.8, which suggests in itself that CYP2C9 may have a low K_m , low V_{max} binding component, and a high K_m , high V_{max} binding component. As a result, data were fit to a two-site biphasic equation instead of the Michaelis-Menten equation. However, naproxen demethylation in the presence of 100 μM dapsone was best fit with the Michaelis-Menten equation (Table 2.2). The inset in Figure 2.8 demonstrates that addition of 100 μM dapsone normalizes naproxen kinetics, resulting in a linear Eadie-Hofstee plot and supporting a fit using the standard Michaelis-Menten equation. This change in kinetic profile is most likely due to dapsone binding to the high K_m site when present in high concentrations, occupying that site such that only one binding region of CYP2C9 is operable in naproxen metabolism. This idea is further supported by the fact that although the Cl_{int} of the second binding site (V_{max2}/K_{m2}) did not change, it did contribute to a lesser extent to the kinetic profile of naproxen in the presence of dapsone (Table 2.2). Piroxicam metabolism is apparently also activated by dapsone, which is interesting considering its structural differences and larger size as compared to naproxen and flurbiprofen. It is worthy to note that piroxicam has been

shown to exhibit substrate inhibition in microsomes and baculovirus-expressed CYP2C9 (unpublished data). However, this was not observed in the expression system used for this study, confirming previous reports that many of these kinetic events are expression system dependent [3].

Fitting of data from all three substrates to the two-site model (equation 2.3) was done to estimate a single K_m and V_{max} , along with the effector binding constant (K_B) and α and β values. Results show α values (change in K_m resulting from effector binding) less than 1, and β values (change in V_{max} resulting from effector binding) greater than 1 for all substrates (Table 2.3), suggesting that K_m was reduced and V_{max} increased in all cases. It has been demonstrated previously that an $\alpha < 1$ and/or a $\beta > 1$ is indicative of activation [2]. It should be realized that the present two-site model equation assumes that the kinetic profile for a substrate's metabolism remains constant, which is not the case for naproxen. However, a model reflecting this would be extremely complex and since no model exists which takes this change into consideration, the results for naproxen must be viewed in this context.

Another component that can be determined when using the two-site model is the binding constant of dapsone. It is apparent from Table 2.3 that this constant varies considerably depending on the substrate being activated, which argues for different binding orientations in the active site or changes in binding efficiency. Other results from this study show that the presence of flurbiprofen does not substantially influence the formation of dapsone hydroxylamine, although conditions were optimized for flurbiprofen hydroxylation, as activation conditions were desired. Close examination of data points in Figure 2.6 indicates an inadequate estimation of V_{max} , which may be the

cause for minimal differences in K_m estimates (81-144 μM). In addition, dapsone has been shown previously to have a sigmoidal kinetic profile [2], suggesting two binding regions for dapsone, one of which is cooperative. However, the sigmoidal portion of dapsone's kinetic profile was present at very low dapsone concentrations, so either not enough low concentration data points were taken in this study to observe sigmoidal kinetics or perhaps the presence of flurbiprofen normalized dapsone kinetics, resulting in a hyperbolic profile. Nonetheless, Michaelis-Menten fits for dapsone hydroxylation in the presence of flurbiprofen appear to be relatively superimposable, suggesting similar kinetic parameters for each condition.

One factor which may affect activation is the presence of cytochrome b_5 which has been shown to affect the catalytic activity of certain P450 reactions [21]. However, our results with baculovirus-expressed CYP2C9 show that activation of flurbiprofen 4'-hydroxylation is not b_5 dependent, as activation still occurs in the absence of cytochrome b_5 (data not shown). It is also possible that dapsone somehow affects the coupling of NADPH-CYP reductase with CYP to increase catalytic activity of CYP2C9, as was suggested by Shou et al. [1] with 7,8-benzoflavone and CYP3A4. Another potential contributor to the activation mechanism may be the interaction with or displacement of water molecules from the enzyme active site, thus affecting hydrogen bonding within the active site. The exit of water molecules from the active site could possibly change the conformation of the enzyme if the hydrogen bonds were in positions essential for stabilizing the protein structure [3]. It is possible that one, all or even additional factors are contributing to some degree to the activation mechanism of dapsone.

Overall, evidence from this study suggests that dapsone activates the metabolism of multiple substrates of CYP2C9 by binding within the active site in a region that causes positive cooperativity. Thus, studies exploring the CYP2C9 active site will be important, beginning with determining the effects of allelic variants and mutants of CYP2C9 on dapsone activation, which may more conclusively prove that active site binding interactions are of significance. With respect to CYP2C9, the *2 (R144C) and *3 (I359L) allelic variants and F114L mutant have been shown to variably affect the metabolism of certain compounds [22;23], and thus are currently being studied to help elucidate the mechanism of activation. Studies exploring analogs of dapsone to determine structural requirements of activation will be reported in the next chapter.

2.5. Acknowledgments

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Figure 2.1. Structures of Three CYP2C9 Substrates

Structures of the three CYP2C9 substrates studied for activation by dapstone. Arrows indicate site of metabolism.

Table 2.1. Michaelis-Menten Parameter Estimates for Flurbiprofen and Piroxicam

Kinetic parameter estimates for flurbiprofen 4'-hydroxylation and piroxicam 5'-hydroxylation in the presence of dapson. Data were fit to the Michaelis-Menten model (equation 2.1).

Dapsone (μM)	(S)-Flurbiprofen		Piroxicam	
	K_m (SE) (μM)	$V_{\max}$ (SE) (pmol/min/pmol P450)	K_m (SE) (μM)	$V_{\max}$ (SE) (pmol/min/pmol P450)
0	28.9 (4.2)	12.6 (0.5)	183 (56)	0.08 (0.01)
2	24.8 (4.2)	15.0 (0.7)	97.3 (14)	0.07 (0.003)
5	17.6 (2.9)	16.3 (0.8)	64.8 (10)	0.07 (0.002)
10	12.6 (1.2)	17.4 (0.4)	134 (35)	0.11 (0.01)
50	9.80 (1.1)	20.7 (0.6)	53.7 (5.6)	0.17 (0.004)
100	10.0 (1.8)	20.6 (1.0)	49.4 (2.0)	0.20 (0.002)

Figure 2.2. Michaelis-Menten Fits for Flurbiprofen 4'-Hydroxylation in Presence of Dapsone.

Activation of Flurbiprofen 4'-Hydroxylation by Dapsone. Fits were to the Michaelis-Menten Model (Equation 2.1), with parameter estimates shown in Table 2.1.

Figure 2.3. Michaelis-Menten Fits for Piroxicam 5'-Hydroxylation in Presence of Dapsone.

Activation of Piroxicam 5'-Hydroxylation by Dapsone. Fits were to the Michaelis-Menten Model (Equation 2.1), with parameter estimates shown in Table 2.1.

Figure 2.4. Biphaseic Fits for Naproxen Demethylation in Presence of Dapsone.

Activation of Naproxen Demethylation by Dapsone. Fits were to a Biphaseic Model (Equation 2.2), with parameter estimates shown in Table 2.2. Notice the change in kinetic profile from biphasic to Michaelis-Menten at 100 μM Dapsone.

Table 2.2. Biphasic Kinetic Parameter Estimates for Naproxen

Kinetic parameter estimates for naproxen demethylation in the presence of dapsone. Data were fit to a two-site biphasic model (equation 2.2).

Dapsone (μM)	K_m1 (SE) (μM)	$V_{\max}1$ (SE) (pmol/min/pmol P450)	Clint (SE) ($\mu\text{l/min/pmolP450}$)
0	433 (252)	11.7 (4.3)	0.002 (0.002)
2	233 (73.5)	14.9 (2.4)	0.003 (0.001)
5	250 (92.4)	21.8 (4.0)	0.002 (0.002)
10	163 (30.8)	24.9 (2.0)	0.002 (0.001)
50	58.9 (18.0)	27.4 (2.7)	0.007 (0.002)
*100	67.4 (22.5)	34.7 (3.8)	0.002 (0.003)

Clint represents the linear portion of the biphasic curve ($V_{\max}2/K_m2$).

*Also fit to Michaelis-Menten equation ($K_m=80.0 \mu\text{M}$; $V_{\max}=37.0\text{pmol/min/pmolP450}$)

Table 2.3. Two Site Model Kinetic Parameter Estimates for Flurbiprofen, Naproxen and Piroxicam

Kinetic parameter estimates for flurbiprofen hydroxylation, naproxen demethylation, and piroxicam hydroxylation in the presence of dapsone. Data were fit to the two-site model (equation 2.3).

Substrate	K_m (SE) (μM)	$V_{\max}$ (SE) (pmol/min/pmol P450)	K_B (SE) (μM)	α (SE)	β (SE)	R^2
(S)-Flurbiprofen	26.5 (3.4)	12.2 (0.5)	17.6 (3.6)	0.3 (0.06)	1.7 (0.1)	0.99
(S)-Naproxen	593 (128)	16.2 (1.6)	68.7 (23)	0.1 (0.04)	2.4 (0.3)	0.99
Piroxicam	110 (17.1)	0.06 (0.003)	164 (77)	0.2 (0.1)	4.0 (0.3)	0.99

K_m is the Michaelis-Menten constant; $V_{\max}$ is the maximal velocity; K_B is the binding constant for the effector; α is the change in K_m resulting from effector binding; β is the change in $V_{\max}$ resulting from effector binding. An α or β value=1 indicates no change in K_m or $V_{\max}$, respectively.

Figure 2.5. Dapsone Activation of Flurbiprofen 4'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of flurbiprofen 4'-hydroxylation by dapsone in expressed CYP2C9. The surface is the fit to equation 2.3.

Figure 2.6. Dapsone Hydroxylation by CYP2C9

Two-dimensional plots demonstrating minimal change in dapsone hydroxylamine formation in the presence of 2, 5 and 10 μM flurbiprofen. Kinetic parameters were estimated using equation 2.1.

Figure 2.7. Dapsone Activation of Naproxen Demethylation (3-D Plot)

Three-dimensional surface plot showing activation of naproxen O-demethylation by dapsone in expressed CYP2C9. The surface is the fit to equation 2.3.

Figure 2.8. Eadie-Hofstee Plot for Naproxen Demethylation

Eadie-Hofstee plot for (S)-naproxen demethylation in the absence of dapsone showing biphasic kinetic profile. Inset figure shows Eadie-Hofstee plot for (S)-naproxen in the presence of 100 μM dapsone, resulting in a linear kinetic profile.

Figure 2.9. Dapsone Activation of Piroxicam 5'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of piroxicam 5'-hydroxylation by dapsone in expressed CYP2C9. The surface is the fit to equation 2.3.

CHAPTER III

Dapsone Activation of CYP2C9-Mediated Metabolism: Structure Activity Relationships and Kinetics

3.1. Introduction

Flurbiprofen is exclusively metabolized by CYP2C9 [1;2], and thus serves as a useful probe for studying regulation of CYP2C9 activity. Dapsone has previously been shown to activate CYP2C9-mediated flurbiprofen 4'-hydroxylation in human liver microsomes and baculovirus-expressed CYP2C9 [3]. In addition, kinetic studies on the activation of flurbiprofen 4'-hydroxylation, as well as naproxen demethylation and piroxicam 5'-hydroxylation by dapsone have recently been reported [4]. Data from this kinetic study were fit to a two-site effector model equation, showing an increase in $V_{\max}$ and decrease in K_m for the metabolism of these substrates in the presence of dapsone, suggesting the possibility of multiple binding regions within the CYP2C9 active site. In addition, dapsone is metabolized by CYP2C9 at therapeutic concentrations to its hydroxylamine metabolite [5;6]. Thus, dapsone is a substrate for the enzyme it activates, further suggesting its presence in the active site during activation. This situation is analogous to that observed with 7,8-benzoflavone, which activates CYP3A4-mediated phenanthrene metabolism, in addition to being a substrate for CYP3A4 [7].

Many structure-activity relationship studies have been performed in describing substrate and inhibitor interactions with CYP2C9 [8-10]. However, similar studies have yet to be done for potential activators of CYP2C9. In a study exploring activation of CYP3A4, a series of partial or modified structures of 7,8-benzoflavone were tested for their ability to activate or inhibit the metabolism of phenanthrene, chrysene, and benzo[a]pyrene *in vitro* [7] to determine the effector structural features necessary for the activation of CYP3A4. We have conducted an analogous study for the activation of

CYP2C9 by dapsone. We report data from a screen of 16 structural analogs of dapsone incubated at a single concentration with flurbiprofen, naproxen, or piroxicam in human liver microsomes. This was done in an attempt to determine effector structural characteristics necessary for activation of CYP2C9. In addition, ten of these analogs were selected, based on structure and results from the screening study, to perform kinetic studies and determine the overall effects of varying the concentration of analog on the metabolism of these model substrates, potentially gaining insight into the mechanism of activation by dapsone.

3.2. Methods

Chemicals. Acetonitrile and dibasic potassium phosphate were obtained from Fisher Scientific Co. (Pittsburgh, PA). (S)-Flurbiprofen, 4'-hydroxy flurbiprofen, and 2-fluoro-4-biphenyl acetic acid (internal standard) were gifts from Pharmacia Corp. (Kalamazoo, MI). Dapsone, sulfamethoxazole, sulfamethazine and ketoprofen were purchased from Sigma Chemical Co. (St. Louis, MO) and all other dapsone analogs were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were obtained from commercial sources and were of the highest purity available.

Enzyme Preparations. Human liver tissue for preparation of microsomes was obtained through the Liver Tissue Procurement and Distribution System. Based on chart review, none of the patients from whom tissues were obtained was taking any medications known to inhibit or induce the P450s. Microsomal samples were prepared according to established methods [11]. Protein content was measured by the method of Lowry et al. [12] and P450 content by the method of Omura and Sato [13].

Human Liver Microsomal Incubation Conditions. Human liver microsomes (0.2 mg/ml final protein concentration, 1.0 mg/ml for piroxicam) were incubated in the presence of 1 mM NADPH and 100 mM K₂HPO₄, pH 7.4, in a total volume of 200 µl to screen for activation by dapsone analogs (Figure 3.1). These analogs were chosen based on their structural similarity to dapsone, with modifications at various parts of the molecule thought to potentially be important for enzyme interactions, e.g., replacement of the amino group (dapsone) with methyl group (*p*-tolylsulfone). Reactions were carried out for 20 minutes at 37°C with either 10 µM (S)-flurbiprofen, (S)-naproxen, or piroxicam (45 minutes), and 10 µM dapsone or analog concentrations. Samples containing flurbiprofen were quenched with 200 µl acetonitrile containing internal standard (180 ng/ml 2-fluoro-4-biphenylacetic acid), while those containing naproxen were quenched with 200 µl acetonitrile. Next, 40 µl ½-strength H₃PO₄ was added to samples containing flurbiprofen or naproxen, followed by centrifugation at 10,000 rpm for 5 minutes, and 10-50 µl was injected onto HPLC system. Samples containing piroxicam were quenched by addition of 20 µl perchloric acid and immediately placed on ice, followed by centrifugation at 10,000 rpm for 5 minutes, and 100 µl was injected onto the HPLC system. Conditions for HPLC analysis of 4'-hydroxy flurbiprofen, desmethylnaproxen, and 5'-hydroxy piroxicam were as described below. These reactions were shown to be linear with respect to time and protein concentration.

Expressed CYP2C9 Incubation Conditions for Kinetic Studies. Ten dapsone analogs were selected for full kinetic studies and included: dapsone, phenylsulfone, *p*-tolylsulfone, 4-nitrophenylsulfonylaniline (4-NPSA), benzophenone, ketoprofen, dicyclohexylketone, sulfadiazine, sulfamethoxazole, and sulfamethazine. Incubation

mixtures in a 6X6 matrix design contained 1 pmol expressed CYP2C9 (5 pmol for piroxicam) co-expressed with reductase (gift from Camitro Corporation, Menlo Park, CA), with either (S)-flurbiprofen (2-300 μ M), (S)-naproxen (10-1800 μ M), or piroxicam (5-900 μ M) incubated with dapsone or dapsone analog (0-100 μ M) in 50 mM potassium phosphate buffer at pH 7.4. Dapsone and analogs were dissolved in DMSO and added to 0.2 mL incubations such that the final concentration of DMSO was less than 2.5%, and reactions were initiated with 1 mM NADPH and allowed to incubate at 37°C for 20 minutes (45 minutes for piroxicam). Control incubations also contained 2.5% DMSO. Samples were processed as stated above for microsomal incubation conditions.

Analysis of 4'-hydroxy flurbiprofen. 4'-Hydroxyflurbiprofen was analyzed by HPLC with fluorescence detection. The HPLC system consisted of a Waters[®] 501 HPLC pump, a Waters[®] 717 autosampler and a Waters[®] 470 fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm. The mobile phase consisted of acetonitrile:20 mM K₂HPO₄, pH 3.0 (45:55, v/v) pumped at 1 ml/min through a Brownlee Spheri-5[®] C₁₈ 4.6 x 100 mm column. 4'-Hydroxy flurbiprofen and internal standard eluted at approximately 2.2 and 4.8 minutes, respectively.

Analysis of desmethylnaproxen. Desmethylnaproxen was analyzed identically to 4'-hydroxy flurbiprofen except that the fluorescence detector was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm, and no internal standard was used. Desmethylnaproxen eluted at approximately 2.1 minutes.

Analysis of 5'-hydroxy piroxicam. 5'-Hydroxy piroxicam was analyzed by HPLC with UV detection (365 nm). The HPLC system was as described above, but was fitted to a Waters[®] 486 UV detector. The mobile phase consisted of 50:50 (v/v), acetonitrile:50

mM K₂HPO₄, pH 3.0 pumped at 1 mL/min through a Phenomenex Luna C₁₈ 5 µm, 4.6 x 150 mm column. Retention time for 5'-hydroxy piroxicam was approximately 4.3 minutes.

Data Analysis and Equations. Kinetic data were fit to a two-site model [3] where appropriate, allowing surface plot analysis and estimation of effector-related changes on substrate K_m and V_{max} of flurbiprofen, naproxen, and piroxicam with the following equation:

$$v = \frac{V_{\max} \bullet [S]}{K_m \left(\frac{1 + \frac{[B]}{K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right) + [S] \left(\frac{1 + \frac{[B]}{\alpha K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right)} \quad (3.1)$$

where S is substrate, B is effector, V_{max} and K_m are kinetic constants for substrate metabolism, K_B is the binding constant for the effector, α is the change in K_m resulting from effector binding, and β is the change in V_{max} from effector binding. For activation, α<1 and/or β>1. Percent activation/inhibition by dapsone analogs was calculated as the mean ± S.D. from incubations run in triplicate. Flurbiprofen and piroxicam data that exhibited little consistent change in presence of effector (resulting in negative or unreasonable parameter estimates when fit to equation 1) were fit to the Michaelis-Menten equation to estimate effector-related changes on metabolism:

$$v = \frac{V_m \bullet S}{K_m + S} \quad (3.2)$$

Naproxen data, again exhibiting little consistent change, were fit to the following biphasic kinetic equation to estimate effector-related changes on metabolism:

$$v = \frac{(V_{m1} \bullet S) + Cl_{int} \bullet S^2}{(K_{m1} + S)} \quad (3.3)$$

where Cl_{int} is the intrinsic clearance, thought to be associated with a second binding region within the active site, and represents the linear portion of the biphasic kinetic curve.

3.3. Results

Dapsone Analogs. Dapsone has previously been shown to activate flurbiprofen 4'-hydroxylation in liver microsomes [3]. Thus, in the present screening study, dapsone was included as a positive control and reference compound. Flurbiprofen 4'-hydroxylation was activated in human liver microsomes ~1.6 fold by dapsone, ~1.4 fold by phenylsulfone, and ~1.5 fold by *p*-tolylsulfone (Figure 3.2). All other analogs either had no effect or were weak inhibitors. Similar results were obtained in preparations from two other human livers (data not shown). Interestingly, only dapsone activated naproxen demethylation (Figure 3.3), while most analogs were weak inhibitors. However, diphenylcyclopropenone was a relatively strong inhibitor of naproxen demethylation (~50%). In addition, the activation by dapsone was almost 3-fold, substantially more than for flurbiprofen metabolism. For piroxicam hydroxylation, dapsone produced ~1.3-fold activation, with phenylsulfone and *p*-tolylsulfone producing similar levels of activation (Figure 3.4). It is interesting to note that these same analogs activated flurbiprofen hydroxylation, albeit to differing degrees. Of the analogs screened, the inhibitor 4-NPSA is perhaps the most interesting, considering its structural similarity to dapsone, phenylsulfone, and *p*-tolylsulfone, all of which activated flurbiprofen and piroxicam hydroxylation.

Kinetic Studies. Analogs for full kinetic studies were selected based on their different functional groups and spatial orientations compared to dapsone, as well as results observed in the initial screen. Experimental data were fit to a two-site effector model (equation 3.1) where appropriate. As has been previously shown (chapter 2), data from dapsone activation of flurbiprofen hydroxylation fits well to this model (Figure 3.5), with an $\alpha = 0.31$, and a $\beta = 1.73$, indicating a decrease in K_m and increase in V_{max} , respectively (Table 3.1). Similarly, phenylsulfone activated flurbiprofen 4'-hydroxylation (Figure 3.6), with an $\alpha = 0.42$, and a $\beta = 2.80$ (Table 3.1). Analogs for which parameters were not estimated from equation 1 due to lack of consistent activation with increasing concentration of effector, inhibition, or lack of effect on flurbiprofen 4'-hydroxylation included *p*-tolylsulfone, benzophenone, ketoprofen, dicyclohexylketone, sulfamethoxazole, sulfadiazine, and sulfamethazine. 4-NPSA, which contains a nitro group in the place of an amino group, acts as a good inhibitor of flurbiprofen hydroxylation (Table 3.2), with Dixon plot analysis suggesting a K_i of approximately 40 μ M (Figure 3.7).

With regard to activation of naproxen demethylation, a good fit to the two-site effector model (equation 3.1) in the presence of dapsone was observed (Figure 3.8), with an $\alpha = 0.09$ and $\beta = 2.40$ (Table 3.1), indicating a substantially greater decrease in K_m and increase in V_{max} (activation) compared to flurbiprofen. Similar to analog screening data, no other analog produced activation comparable to dapsone, although both *p*-tolylsulfone and sulfamethazine activated at high concentrations (Figures 3.9 and 3.10), with an increase in V_{max} noted in the presence of effector (Table 3.3). Interestingly, both phenylsulfone and 4-NPSA inhibited naproxen demethylation (Figures 3.11 and 3.12),

with kinetic parameters shown in Tables 3.4 and 3.5, respectively. Inhibition by 4-NPSA was pronounced only at high concentrations. Analogs for which parameters were not estimated included benzophenone, ketoprofen, dicyclohexylketone, sulfadiazine, and sulfamethoxazole, as no measurable effect was observed at any concentration. Table 3.1 also shows the kinetic parameters for activation of the metabolism of piroxicam, a structurally distinct CYP2C9 substrate. Similar to flurbiprofen, piroxicam 5'-hydroxylation in the presence of dapson (Figure 3.13) and phenylsulfone (Figure 3.14) gave α and β parameter estimates indicative of activation (Table 3.1), while benzophenone activated at high concentrations (Figure 3.15), with an increase in both $V_{\max}$ and K_m (Table 3.6). Interestingly, 4-NPSA completely inhibited formation of 5'-hydroxy piroxicam. All other analogs had minimal effects on piroxicam 5'-hydroxylation.

3.4. Discussion

It has previously been observed that CYP2C9 displays atypical kinetics, as evidenced by dapson activation of CYP2C9-mediated flurbiprofen 4'-hydroxylation [3]. In addition, the kinetic effects of this activation on the metabolism of three prototypical CYP2C9 substrates (flurbiprofen, naproxen, and piroxicam) have been reported [4]. To gain insight into activation by dapson, sixteen analogs of dapson were studied with these same three substrates to determine structural requirements for activation of CYP2C9-mediated metabolism. Additionally, ten of these analogs were selected for kinetic studies to determine the effect of varying the concentrations of effector on CYP2C9 activity. Screening results suggest that the level of activation is substrate

dependent, and that a sulfone moiety between two benzene rings is necessary for activation of CYP2C9. Activation of naproxen demethylation appears to be the most restrictive, as only dapsone was able to consistently activate this metabolic event. Kinetic studies produced similar results, although certain analogs exhibited effects at high concentrations.

In terms of atypical kinetic behavior, CYP3A4 has been the most studied isoform. Several studies have shown that purified CYP3A4 enzymes are directly activated by 7,8-benzoflavone [7;14]. This is particularly interesting since 7,8-benzoflavone is a substrate for the enzyme that it activates, analogous to dapsone and CYP2C9 [3;4], suggesting similar activation characteristics between these isoforms. In addition, Shou et al. have explored the structural requirements for activation of CYP3A4 by studying the effects of structural analogs of 7,8-benzoflavone on CYP3A4 activity [7]. To this end, similar studies have been performed for CYP2C9. Experimental data from dapsone analogs suggest that some very strict structural constraints are necessary for activation of CYP2C9, as dapsone, phenylsulfone, and *p*-tolylsulfone activated flurbiprofen and piroxicam metabolism (Figures 3.2 and 3.4, respectively), while only dapsone activated naproxen demethylation (Figure 3.3). The common functional group within these activators is a sulfone group directly between two benzene rings. Replacement of this sulfone with a carbonyl (benzophenone), sulfide (diphenylsulfide), sulfoxide (diphenylsulfoxide), or sulfilimine (diphenylsulfilimine) functional group resulted in elimination of activation properties, suggesting that the sulfone group directly between two benzene rings is vital for binding to an effector site region that results in positive cooperativity (activation) of CYP2C9. In fact, some analogs were inhibitors of CYP2C9-

mediated metabolism. Interestingly, if one of the terminal amino groups on dapsone is replaced by a nitro (4-NPSA), then the analog becomes an inhibitor, whereas replacement of both terminal amino groups by two methyl groups (*p*-tolylsulfone) or left vacant (phenylsulfone) had little effect on the ability to activate flurbiprofen and piroxicam. With respect to naproxen, only dapsone activated its demethylation, with a substantially higher degree of activation than either flurbiprofen or piroxicam hydroxylation. This suggests even greater effector structural requirements for activation of naproxen metabolism. It is hypothesized that naproxen may have a different binding orientation than either flurbiprofen or piroxicam, resulting in slightly different requirements for activation of its metabolism. In addition, other analogs with two benzene rings, two cyclohexyl groups or the sulfone moiety in a different spatial arrangement (sulfonamide analogs) generally resulted in inhibition, further arguing for the direct association of a sulfone group with two benzene rings for activation.

Analogs for kinetic studies were selected based on results from screening data, in addition to selection based on variety of functional group composition. Kinetic data generally agreed with the analog screening data, with activation by dapsone noted for all three reporter substrates. In addition, data for phenylsulfone activation of flurbiprofen and piroxicam metabolism fit well to the two-site effector model, while no other analog substantially activated naproxen demethylation. However, for some analogs, activation was observed at higher effector concentrations (Figures 3.9, 3.10, and 3.15), signifying a lower affinity of the “effector site” for some analogs. Of these, benzophenone activation of piroxicam 5'-hydroxylation at high concentrations (Figure 3.15) was the most intriguing, considering it was the only analog without a sulfone group that caused any

type of activation. The absence of the sulfone group may have been responsible for the lowered affinity for benzophenone, resulting in no effect at low concentrations. In addition, there were examples in which increasing concentrations of effector had variable effects on the substrate's metabolism (i.e. initial increase then a decrease in metabolism, or vice versa). This concentration dependent observation, which resulted in an inability to fit data to the two-site effector model (equation 3.1), has been noted by other groups, as well. Wang et al. observed that low concentrations of midazolam slightly activated terfenadine metabolism in human liver microsomes, but inhibited this pathway at higher concentrations [15]. The possibility should also be mentioned that some of the analogs may elicit their effects by other means than binding to the effector site, contributing to this variability. An example in which the screening and kinetic data did not coincide included the effect of *p*-tolylsulfone on flurbiprofen 4'-hydroxylation, as well as on naproxen demethylation. Screening data at a single concentration suggested that this analog activated flurbiprofen 4'-hydroxylation (Figure 3.2), and had no effect on naproxen demethylation (Figure 3.3). Meanwhile, kinetic data obtained using multiple substrate and effector concentrations suggested minimal changes in flurbiprofen 4'-hydroxylation (data not shown), while naproxen demethylation was activated at high concentrations of *p*-tolylsulfone (Figure 3.9). It is possible that some of this incongruency may be due to the different source of enzyme used for the two studies (human liver microsomes vs. expressed CYP2C9 microsomes). In addition, kinetic studies suggest that phenylsulfone acts as an inhibitor of naproxen demethylation, eventually changing the kinetic profile from biphasic to hyperbolic at 100 μ M (Figure 3.11), while it clearly activates both flurbiprofen and piroxicam hydroxylation (Figures 3.6 and 3.14). The

observed change in naproxen kinetic profile from biphasic to hyperbolic in the presence of phenylsulfone is similar to that observed for dapsone (chapter 2), only inhibition is the end result instead of activation. A clear explanation for this is not altogether apparent at this time. It is possible that naproxen binds in a different orientation than either flurbiprofen or piroxicam, resulting in the difference in observed effects. In fact, naproxen has been shown to display biphasic kinetics [3;4;16], suggesting multiple binding orientations for itself, which may contribute to this difference.

One of the most intriguing analogs is 4-NPSA, which inhibited both flurbiprofen ($K_i \sim 40 \mu\text{M}$) and naproxen (K_i not estimated due to biphasic nature) metabolism, while completely eliminating piroxicam hydroxylase activity. The similarities in structure of 4-NPSA to dapsone, and yet the substantial differences in effect on CYP2C9-mediated metabolism are striking. Possible explanations for these observations include steric and electronic effects of the nitro group compared to the amino group. Many studies have suggested that the active site of CYP2C9 has positive residues which interact with negatively charged groups on substrates [17-20]. Perhaps some type of electrostatic interaction between the nitro group of 4-NPSA and one of these active site residues may orient it in such a way as to cause inhibition rather than activation. Dixon plot analysis for flurbiprofen hydroxylation suggests that 4-NPSA is acting in a noncompetitive manner, for which binding of substrate and inhibitor is not mutually exclusive. Another interesting observation is that of the effect of *p*-tolylsulfone and sulfamethazine at high concentrations on naproxen demethylation, showing a minimal effect on K_m but a rather substantial effect on V_{max} (Table 3.3). A similar effect on piroxicam 5'-hydroxylation was observed at high concentrations of benzophenone (Table 3.6). Reasons for the direct

effect on $V_{\max}$, with minimal effect on K_m could be numerous. One possibility may be alteration of uncoupling mechanisms by the effector, most notably a decrease in hydrogen peroxide release due to exclusion of water molecules from the active site by the effector, resulting in enhanced catalytic activity [21]. Another possibility may be that the effector enhances the interaction of CYP450 with cytochrome b5, which has been shown to decrease uncoupling to hydrogen peroxide formation, thereby increasing the concentration of active oxygen species for catalysis [22].

Overall, analog results for activation of CYP2C9 suggest that specific activator-protein interactions may be involved in the recognition and binding of effector, as even small deviations from the core structure of dapson resulted in variable effects or inhibition. Results from this study do not conclusively establish whether the effector site lies within or separate from the active site. Although the location of the effector site has not been conclusively proven, many models have been proposed. Ueng et al. [23] have described a model suggesting that an allosteric site is involved in the cooperativity of certain CYP3A4 reactions, but the proximity of this site is not defined. Harlow and Halpert suggest that effector binding is most likely in the active site along with the substrate, as they were able to create an active site double mutant of CYP3A4 that reduced positive cooperativity in the presence of 7,8-benzoflavone[24]. Another model proposed by Shou and Korzekwa suggests that both substrate and effector are bound in the active site simultaneously, each having a distinctive binding region and access to the reactive oxygen [3;7]. Recently, it was reported that this two-site binding model applies to CYP2C9 [4]. However, the most appropriate model may be dependent on the substrate and effector in question. In addition, there is the possibility of conformational changes

due to binding of effector, for which these models can not predict. Therefore, one of the most reasonable explanations for the cause of atypical kinetics is one in which the multiple binding-site model and a conformationally based model are combined, as suggested by Atkins et al. [25]. Regardless of the most appropriate model, kinetic data from previous studies suggest that the effector site for CYP2C9 is within the active site [4]. Therefore, it is important to more fully characterize critical active site binding regions and residues that may be involved in binding of dapsone and/or some of its analogs. He et al. have suggested that the CYP2C9 active site has two regions that bind electronegative groups, one of which may be the sulfone group of dapsone [19]. In addition, the positive arginine residues at positions 97 and 105 have been shown to be important in the catalytic function of CYP2C9 [20]. It would be interesting to mutate these to neutral residues and observe if there is any effect on activation by dapsone. In similar studies, Domanski et al. selected certain residues to mutate based on sequence alignments of CYP3A4 with bacterial enzymes, and have been able to demonstrate which residues are most likely involved in 7,8-benzoflavone activation of steroid hydroxylation [26;27]. This group concluded that progesterone and 7,8-benzoflavone bind at separate locations within the CYP3A4 active site, even though they both appear to have equal access to the reactive oxygen. A reasonable starting point for CYP2C9 would be to determine the effects of allelic variants and mutants of CYP2C9 on activation, which may more conclusively prove that active site binding interactions are of substantial importance. The *2 and *3 allelic variants and F114L mutant have been shown to variably affect the metabolism of certain compounds [28;29], suggesting similar characteristics as those observed with mutants of CYP3A4. More specifically, the *2

allele change shows evidence of affecting the interaction of the P450 with oxidoreductase [30], which would affect electron flow and perhaps the activation process. Studies exploring the critical residues of CYP2C9 involved in binding of various substrates and activation by dapsone have been performed and will be reported in the next chapter.

3.5. Acknowledgments

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Figure 3.1. Structures of Dapsone and Analogs

Dapsone and analogs examined for effect on flurbiprofen, naproxen, and piroxicam metabolism.

Figure 3.2. Screen of Dapsone Analog Effect on Flurbiprofen 4'-Hydroxylation

Activation/inhibition of flurbiprofen 4'-hydroxylation by dapsone and analogs.

Incubations were performed in triplicate with 10 μ M flurbiprofen and 10 μ M effector (dapsone or analog).

Figure 3.3. Screen of Dapsone Analog Effect on Naproxen Demethylation

Activation/inhibition of naproxen demethylation by dapsone and analogs. Incubations were performed in triplicate with 10 μ M flurbiprofen and 10 μ M effector (dapsone or analog).

Figure 3.4. Screen of Dapsone Analog Effect on Piroxicam 5'-Hydroxylation

Activation/inhibition of piroxicam 5'-hydroxylation by dapsone and analogs. Incubations were performed in triplicate with 10 μ M flurbiprofen and 10 μ M effector (dapsone or analog).

Table 3.1. Two-Site Kinetic Parameter Estimates for Dapsone and Analogs

Kinetic parameters (SE) derived from two-site effector model (equation 3.1) for activation of CYP2C9-mediated metabolism.

Flurbiprofen 4'-hydroxylation

Analog	$V_{\max}$	K_m	K_B	α	β	R^2
Dapsone	12.2 (0.6)	26.5 (3.4)	17.6 (3.6)	0.31 (0.06)	1.73 (0.09)	0.99
Phenylsulfone	6.5 (0.6)	26.0 (6.1)	113.3 (98.7)	0.42 (0.28)	2.80 (0.44)	0.95

Naproxen Demethylation

Dapsone	16.2 (1.6)	593.4 (128.2)	68.7 (22.7)	0.09 (0.03)	2.40 (0.26)	0.99
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Piroxicam 5'-hydroxylation

Dapsone	0.07 (0.01)	22.6 (10.5)	76.5 (73.4)	0.45 (0.45)	5.92 (0.94)	0.94
Phenylsulfone	0.08 (0.005)	31.3 (6.1)	299.2 (298.9)	0.18 (0.17)	4.41 (0.38)	0.98

$V_{\max}$ is in pmol/min/pmol P450; K_m and K_B are in μM

Figure 3.5. Dapsone Activation of Flurbiprofen 4'-hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of flurbiprofen 4'-hydroxylation by dapsone. The surface is the fit to equation 3.1, and is the same as seen in chapter 2.

Figure 3.6. Phenylsulfone Activation of Flurbiprofen 4'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of flurbiprofen 4'-hydroxylation by phenylsulfone. The surface is the fit to equation 3.1.

Figure 3.7. 4-NPSA Inhibition of Flurbiprofen 4' Hydroxylation (Dixon Plot)

Dixon plot suggestive of noncompetitive inhibition of flurbiprofen 4'-hydroxylation by 4-NPSA, with a $K_i \sim 40 \mu\text{M}$.

Table 3.2. 4-NPSA Inhibition of Flurbiprofen 4' Hydroxylation

Kinetic parameters (SE) for flurbiprofen 4'-hydroxylation estimated from Michaelis-Menten equation (equation 3.2) in presence of 4-NPSA.

	0μM	2μM	5μM	10μM	50μM	100μM
$V_{\max}$	9.0 (0.7)	8.3 (0.4)	7.6 (0.3)	7.4 (0.6)	5.3 (0.3)	3.0 (0.3)
K_m	31.0 (8.0)	23.0 (4.0)	19.8 (2.6)	39.0 (10.6)	27.8 (5.4)	28.8 (8.7)

$V_{\max}$ =pmol/min/pmol P450; K_m =μM

Figure 3.8. Dapsone Activation of Naproxen Demethylation (3-D Plot)

Three-dimensional surface plot showing activation of naproxen demethylation by dapsone. The surface is the fit to equation 3.1, and is the same as seen in chapter 2.

Figure 3.9. Activation of Naproxen Demethylation by *p*-Tolylsulfone

Activation of naproxen demethylation at high concentrations of *p*-tolylsulfone. Fits were to a biphasic kinetic equation (equation 3.3). Notice the change toward a more hyperbolic function in the presence of 100 μM *p*-tolylsulfone.

Figure 3.10. Activation of Naproxen Demethylation by Sulfamethazine

Activation of naproxen demethylation at high concentrations of sulfamethazine. Fits were to a biphasic kinetic equation (equation 3.3).

Table 3.3. Activation of Naproxen Demethylation by p-Tolysulfone and Sulfamethazine

Kinetic parameters (SE) for naproxen demethylation estimated from biphasic kinetic equation (equation 3.3) showing activation by *p*-tolylsulfone and sulfamethazine at 50 and 100μM.

<p>p-Tolylsulfone</p>	0μM	50μM	100μM
V _{max} 1	2.8 (0.6)	19.2 (4.1)	21.8 (4.4)
K _m 1	45.7 (26.5)	130.5 (70.8)	67.0 (54.0)
Clint	0.01 (.0003)	0.003 (.002)	0.003 (.003)

<p>Sulfamethazine</p>	0μM	50μM	100μM
V _{max} 1	7.9 (0.4)	8.1 (0.4)	11.1 (0.9)
K _m 1	208.6 (22.9)	155.7 (15.5)	233.8 (44.1)
Clint	0.003 (.0002)	0.004 (.0002)	0.002 (.0004)

V_{max} 1=pmol/min/pmol P450; K_m1=μM; Clint=V_{max}2/K_m2
(μl/min/pmol P450)

Figure 3.11. Inhibition of Naproxen Demethylation by Phenylsulfone

Biphasic kinetic plots showing inhibition of naproxen demethylation by phenylsulfone. Fits were to a biphasic kinetic equation (equation 3.3). Note the change in profile to hyperbolic function in presence of 100 μM phenylsulfone.

Table 3.4. Inhibition of Naproxen Demethylation by Phenylsulfone

Kinetic parameters (SE) for naproxen demethylation estimated from biphasic kinetic equation (equation 3.3) showing inhibition by phenylsulfone.

Phenylsulfone	0 μM	2 μM	5 μM	10 μM	50 μM	100 μM
$V_{\max 1}$	4.9 (1.7)	3.8 (0.1)	2.8 (0.6)	3.2 (1.4)	1.9 (0.1)	2.2 (0.3)
K_m1	140.5 (93.4)	52.4 (5.3)	34.2 (25.3)	53.7 (70.1)	3.7 (1.6)	2.3 (3.1)
Clint	0.008 (.0006)	0.005 (.0001)	0.004 (.0004)	0.003 (.001)	0.002 (.0001)	0.001 (.0003)

$V_{\max 1}$ =pmol/min/pmol P450; K_m1 = μM ; Clint= $V_{\max 2}/K_m2$ ($\mu\text{l/min/pmol P450}$)

Figure 3.12. Inhibition of Naproxen Demethylation by 4-NPSA

Biphasic kinetic plots showing inhibition of naproxen demethylation by 4-NPSA, particularly at 100 μM. Fits were to a biphasic kinetic equation (equation 3.3).

Table 3.5. Inhibition of Naproxen Demethylation by 4-NPSA

Kinetic parameters (SE) for naproxen demethylation estimated from biphasic kinetic equation (equation 3.3) showing inhibition by 4-NPSA, most notably at 100μM.

0μM	10μM	100μM
V_{max1}	15.9 (7.1)	11.2 (4.4)	1.4 (0.2)
K_m1	695 (427.6)	586 (320.6)	124.3 (40.2)
Clint	0.002 (.002)	0.003 (.001)	0.001 (.0001)

V_{max1} =pmol/min/pmol P450; K_m1 =μM; Clint= V_{max2}/K_m2 (μl/min/pmol P450)

Figure 3.13. Dapsone Activation of Piroxicam 5'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of piroxicam 5'-hydroxylation by dapsone. The surface is the fit to equation 3.1 and is the same as seen in chapter 2.

Figure 3.14. Phenylsulfone Activation of Piroxicam 5'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of piroxicam 5'-hydroxylation by phenylsulfone. The surface is the fit to equation 3.1.

Figure 3.15. Activation of Piroxicam 5'-Hydroxylation by Benzophenone

Michaelis-Menten plots showing activation of piroxicam 5'-hydroxylation by benzophenone at 50 and 100 μM . Fits were to the Michaelis-Menten equation (equation 3.2).

Table 3.6. Activation of Piroxicam 5'-Hydroxylation by Benzophenone

Kinetic parameters for piroxicam 5'-hydroxylation estimated by Michaelis-Menten equation (equation 3.2) showing activation by benzophenone at 50 and 100 μM .

Benzophenone	0 μM	50 μM	100 μM
V_{max}	0.1 (.005)	0.17 (.005)	0.21 (.01)
K_m	74.8 (13.8)	85.5 (9.3)	121.5 (19.1)

V_{max} =pmol/min/pmol P450; K_m = μM

CHAPTER IV

Dapsone Activation of CYP2C9 Allelic Variants

4.1. Introduction

Many studies have suggested that certain CYP450 enzymes may behave in a non Michaelis-Menten or atypical fashion [1-3]. Our lab has demonstrated previously that dapsone activates CYP2C9-mediated flurbiprofen 4'-hydroxylation in microsomes and purified enzyme [2]. More recently, we have described the kinetic effects of dapsone activation of flurbiprofen metabolism, as well as effector structural requirements for this activation. In these studies, data supported a two-site model, which suggested that dapsone and flurbiprofen bind to distinctive regions within the active site simultaneously, each having access to the active oxygen, and thus being metabolized. Therefore, it is hypothesized that binding interactions within the active site are of prime importance to the CYP2C9 activation mechanism, which has yet to be fully characterized.

Allelic variants of CYP2C9 (*2 (R144C) and *3 (I359L)) along with the F114L mutant have been shown to variably affect the metabolism of certain compounds [4-8]. These differences in metabolism likely result from these residues being in a position important for either substrate binding, protein conformation, or perhaps electron flow to the P450, and thus may be an important factor in the activation mechanism. The purpose of the current study was to determine the effects of dapsone on the metabolism of the CYP2C9 substrates (S)-flurbiprofen [9;10], (S)-naproxen [11;12], and piroxicam [13] when incubated with wild-type CYP2C9, allelic variants of CYP2C9, or the F114L mutant. Wang et al. [14] suggested that *in vitro* drug-drug interaction patterns with CYP3A4 are substrate dependent, giving credence to analogous studies with CYP2C9. These three substrates potentially have different binding orientations in the CYP2C9 active site, perhaps resulting in differences in the ability to be metabolically activated. In

addition, effects of dapsone on flurbiprofen kinetics in allelic variants and the F114L mutant were measured to better understand the CYP2C9 enzyme structural requirements necessary for activation.

By observing the effects of these variants and mutant on dapsone activation of various CYP2C9 substrates, insight may be gained into the active site interactions necessary for activation with respect to each substrate, providing us with mechanistic details for dapsone activation of CYP2C9.

4.2. Methods

Chemicals. Acetonitrile and dibasic potassium phosphate were obtained from Fisher Scientific Co. (Pittsburgh, PA). (S)-flurbiprofen, 4'-hydroxyflurbiprofen, and 2-fluoro-4-biphenylacetic acid (internal standard) were gifts from Pharmacia Corp. (Kalamazoo, MI). (S)-naproxen and desmethylnaproxen were gifts from Syntex Laboratories Inc. (Palo Alto, CA). Piroxicam and dapsone were purchased from Sigma Chemical Co. (St. Louis, MO), and 5'-hydroxy piroxicam was a gift from Pfizer Inc. (Groton, CT.). CYP2C9 allelic variant and F114L mutant enzymes were provided by Dr. Allan Rettie of University of Washington. All other chemicals were obtained from commercial sources and were of the highest purity available.

Enzyme Preparation. Baculovirus-mediated CYP2C9 expression and purification from insect cells were carried out by growth and passage of *Trichopulsia ni* (Gibco BRL) cells using the HyQCCM-3 medium (HyClone Laboratories). Cotransfection of *T. ni* cells with Bsu361-digested BacPAK6 viral DNA and the appropriate CYP2C9 mutant transfer vector was carried out with the reagents and procedures provided by Clontech Laboratories, Inc. for construction and amplification of recombinant viral stocks. Cells

were pelleted 2-3 days postinfection, resuspended, washed once in glycerol-containing buffer (100 mM potassium-phosphate, 20% glycerol, 0.33 mM DTT, and 1 mM EDTA (pH 7.4)), repelleted, and stored at -80°C until further use. Cultures exhibiting expression levels of 75 nmol/L were used for enzyme purification. Purification of enzyme was achieved from detergent-solubilized *T. ni* cell pellets using sequential chromatography on Octyl-Sepharose, DEAE-Sepharose, and ceramic hydroxyapatite to specific contents in excess of 10 nmol of holoenzyme/mg of protein for each enzyme.

Purified CYP2C9 Enzyme Incubation Conditions. All purified P450 enzymes were reconstituted with P450 reductase (PanVera, Madison, WI) and cytochrome b5 (PanVera, Madison, WI) in a 1:2:1 ratio with dilauroylphosphatidylcholine vesicles (10 µg) extruded through a 200 nm pore-sized membrane. The reconstituted enzymes were incubated in the presence of either 10 µM (S)-flurbiprofen, (S)-naproxen, or piroxicam with 10 µM dapsone in 50 mM potassium phosphate buffer, pH 7.4 in a final volume of 200 µL. Reactions were initiated by the addition of 1 mM NADPH and carried out for 20 minutes (piroxicam for 45 minutes) at 37°C. Kinetic studies involving (S)-flurbiprofen and dapsone were performed as described above with (S)-flurbiprofen concentrations ranging from 2-150 µM. Dapsone concentrations were 0, 2, and 5 µM. All reactions were shown to be linear with respect to time and protein concentration.

Analysis of 4'-hydroxy flurbiprofen. 4'-Hydroxy flurbiprofen was analyzed by HPLC with fluorescence detection. Samples were quenched with 200 µL of acetonitrile containing 180 ng/mL of 2-fluoro-4-biphenylacetic acid (internal standard) and acidified with 40 µL ½-strength phosphoric acid. Samples were then centrifuged at 10,000 rpm for 5 minutes and 10-50 µL injected onto the HPLC system. The HPLC system consisted of

a Waters[®] 501 HPLC pump, a Waters[®] 717 autosampler and a Waters[®] 470 fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm. The mobile phase consisted of acetonitrile:20 mM K₂HPO₄, pH 3.0 (40:60) pumped at 1 mL/min through a PE-Brownlee Spheri-5 C₁₈ 4.6 x 100 mm column. 4'-hydroxy flurbiprofen and internal standard eluted at approximately 3.5 and 9.0 minutes, respectively.

Analysis of desmethylnaproxen. Desmethylnaproxen was analyzed identically to 4'-hydroxy flurbiprofen except that the fluorescence detector was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm, and no internal standard was used. Desmethylnaproxen eluted at approximately 2.1 minutes.

Analysis of 5'-hydroxy piroxicam. 5'-Hydroxy piroxicam was analyzed by HPLC with UV detection (365 nm). Samples were quenched with 20 µL of perchloric acid, vortexed and placed on ice. Samples were then centrifuged at 10,000 rpm for 5 minutes and 100 µL injected onto HPLC system. The HPLC system was as described above, but was fitted to a Waters[®] 486 UV detector. The mobile phase consisted of acetonitrile:50 mM K₂HPO₄, pH 3.0 (50:50) pumped at 1 mL/min through a Phenomenex Luna C₁₈ 5 µm, 4.6 x 150 mm column. Retention time for 5'-hydroxy piroxicam was approximately 4.3 minutes.

Data Analysis. Velocities were calculated as the mean of incubations done in triplicate. Kinetic parameters were estimated following nonlinear regression using Sigma Plot (Version 6.0).

4.3. Results

Dapsone activation of flurbiprofen, naproxen, and piroxicam in CYP2C9 variants. Activation of flurbiprofen, naproxen, and piroxicam (Figure 4.1) metabolism by dapsone was determined in purified CYP2C9*1 (wild-type), *2 (R144C) and *3 (I359L) variants, and F114L mutant by incubating each particular substrate at 10 μ M with 10 μ M dapsone in triplicate. Flurbiprofen hydroxylation was activated about 2-fold in the presence of dapsone for CYP2C9 wild-type, as well as *2 and *3 variants (Figure 4.2, A). However, in the F114L mutant, flurbiprofen was activated almost 5-fold (Figure 4.2, A). Naproxen demethylation (Figure 4.2, B), was activated by dapsone almost 6-fold in the wild-type enzyme, substantially higher than observed for flurbiprofen. The *2 variant and F114L mutant displayed about the same level of activation as observed with wild-type enzyme, while only 1.5-fold activation was observed in the *3 variant. Results of piroxicam hydroxylation (Figure 4.2, C) demonstrate an approximately 2-fold activation in the wild-type enzyme, with slightly higher activation in the *2 variant. Activation in the *3 variant could not be determined due to low initial activity in the absence of dapsone, although detectable activity was subsequently observed when dapsone was added. Interestingly, no activation of piroxicam hydroxylation by dapsone was noted in the F114L mutant even though the turnover of piroxicam by this mutant was 2-fold higher than observed with wild-type enzyme.

Flurbiprofen kinetic studies. To more completely assess this activation phenomenon, kinetic studies were carried out to determine the effect of dapsone on flurbiprofen 4'-hydroxylation in wild-type CYP2C9, its variants and the F114L mutant. Kinetic parameter estimate results (Table 4.1) indicate that dapsone activates flurbiprofen

4'-hydroxylation in all enzymes tested, increasing $V_{\max}$, while lowering K_m . Two-dimensional plots exhibiting this activation are presented in Figures 4.3-4.6, with inset Lineweaver-Burke plots indicative of ternary complex formation, as oppose to the ping-pong model. Only the Lineweaver-Burke plot for the *3 variant did not show ternary complex formation. For wild-type CYP2C9, *2 variant, and F114L mutant, $V_{\max}$ was increased only slightly. However, K_m values were substantially reduced with increasing dapsone concentration. Conversely, the $V_{\max}$ for flurbiprofen 4'-hydroxylation in the *3 variant almost doubled in the presence of 5 μM dapsone, although initial activity was considerably lower. The most substantial effect on K_m was observed with the F114L mutant, where K_m was reduced from 246 μM to 44 μM by dapsone co-incubation.

4.4. Discussion

The activation of CYP enzymes *in vitro* has been observed for virtually every isoform involved in human drug metabolism. However, little is known about the effect of this activation on substrate binding and in particular, the importance of various critical amino acid residues. To this end, studies were undertaken to identify substrate differences with regard to CYP2C9 activation as well as differences among the common allelic variants of CYP2C9 and a mutant with a substitution made in a critical pi-stacking binding region. In the current study, 10 μM dapsone activated flurbiprofen 4'-hydroxylation about 1.5-fold in wild-type CYP2C9, with similar results observed in the *2 and *3 variants, while almost 5-fold activation was observed in the F114L mutant. Meanwhile, dapsone activation of naproxen demethylation in wild-type CYP2C9 was almost 6-fold, substantially higher than observed for flurbiprofen, while the *2 variant

and F114L mutant exhibited an approximately 5-fold activation. Interestingly, piroxicam 5'-hydroxylation activity in the F114L mutant was almost 2-fold higher than wild-type CYP2C9 in the absence of dapsone. However, no activation was observed in the F114L mutant despite this higher turnover rate, whereas the wild-type *2 and *3 variants all exhibited 2-3-fold activation. Kinetic studies with dapsone and flurbiprofen demonstrated that K_m values for flurbiprofen were decreased in all recombinant CYP2C9 enzymes in the presence of dapsone, with the greatest effect observed for the F114L mutant, while V_{max} estimates generally increased. These results suggest that dapsone binding results in positive cooperativity regarding binding interactions within the active site.

Observations of non Michaelis-Menten or atypical CYP450 kinetics have increased in the area of drug metabolism, with the CYP3A4 isoform being the most studied [1;2;15;16]. Atypical kinetic events such as activation, autoactivation, and substrate inhibition are now being observed in other relevant CYP450 isoforms, suggesting that these observations may be common to all CYP450 isoforms to some extent [2;3]. This *in vitro* phenomenon is particularly relevant because it makes predicting *in vivo* pharmacokinetic behavior very complex [17]. Our lab has demonstrated that CYP2C9-mediated flurbiprofen 4'-hydroxylation is activated by dapsone in human liver microsomes, as well as purified CYP2C9 [2]. We have since demonstrated the full kinetic effects of dapsone on flurbiprofen metabolism [18]. However, the exact mechanism for this activation remains to be defined, although it is believed that dapsone and flurbiprofen are bound in the active site simultaneously, being described by a two-site model describing activation [1;2]. Therefore, active site

properties and binding interactions may be of substantial importance in the mechanism of dapsone activation.

CYP2C9 is polymorphically expressed in humans, with the three major alleles being referred to as CYP2C9*1 (wild-type), *2 (R144C), and *3 (I359L). These allelic variants, along with an F114L mutant affecting the “pi-stacking” region of CYP2C9, have been shown to variably affect the metabolism of certain substrates [4-8]. Studies utilizing these variant enzymes have been instrumental in determining structure-function relationships. Because of these allelic functional differences, it was hypothesized that study of the variants and F114L mutant with various substrates and dapsone would provide insight into binding characteristics and the activation phenomenon.

Within the CYP2C9 active site, it has been suggested that there are six substrate recognition sites (SRS), which variably contribute to the recognition and binding of substrates [19]. While it has been shown that position 144 is not in any known substrate recognition sequence [19], Crespi and Miller [6] suggest that this residue plays an important role in the interaction between CYP2C9 and oxidoreductase. However, position 359 falls within SRS 5, and thus would be expected to affect binding of certain substrates. The F114L mutant is also important in substrate recognition, as it lies within SRS1, and is believed to play a critical role in *pi-pi* stacking interactions between certain aromatic substrates and the active site of CYP2C9 [8]. Therefore, it is likely that these variants and F114L mutant will have an affect on dapsone activation of CYP2C9. Similar studies by Domanski et al. [20;21] have demonstrated the importance of specific active site residues in the cooperativity of CYP3A4 by α -naphthoflavone. Additional evidence through the use of a L211F/D214E double mutant in the analysis of CYP3A4

cooperativity suggests that the effector site is part of the active site [22]. Thus, it is possible that the CYP2C9 effector site is also present in the active site.

While we have shown that dapsone activates flurbiprofen metabolism *in vitro*, it is important to determine how dapsone affects other CYP2C9 substrates. It has been demonstrated with CYP3A4 that *in vitro* drug-drug interactions are substrate dependent [14], and thus, similar observations may be noted with CYP2C9. Naproxen and piroxicam have been shown to be O-demethylated and hydroxylated, respectively, by CYP2C9 [11-13]. As a result, these compounds were chosen as prototypical substrates of CYP2C9 in addition to flurbiprofen. Thus, by determining the effects of dapsone on various substrates of CYP2C9 in allelic variant and mutant enzymes, the role of various active site residues important to the mechanism of activation by dapsone was more fully elucidated.

Experimental results indicate that position 114 in SRS1 is an important determinant of CYP2C9 activation of flurbiprofen and piroxicam. Firstly, flurbiprofen hydroxylation was activated almost 5-fold in the F114L mutant, compared to 1.5-fold activation in wild-type. This suggests that without the phenylalanine residue and the resulting lack of *pi*-stacking interaction in the active site, flurbiprofen can bind in a more productive orientation (less restricted), resulting in enhanced flurbiprofen hydroxylase activity. Alternatively, piroxicam metabolism was not activated in the F114L mutant. In fact, activity was higher in the F114L mutant than wild-type CYP2C9 in the absence of dapsone, suggesting that a *pi*-stacking interaction with the phenylalanine residue perhaps limits maximal piroxicam metabolism. The results from naproxen are interesting in that its metabolism was activated substantially more than either flurbiprofen or piroxicam,

suggesting that naproxen may have a different binding orientation than the other CYP2C9 substrates. This is not completely unexpected since naproxen demethylation exhibits a biphasic kinetic profile suggestive of homotropic cooperativity in and of itself [12]. In addition, there was no substantial difference in the level of activation for the *2 variant and F114L mutant when compared to wild-type, indicating that these residues are not important determinants in the activation of naproxen. Conversely, the *2 variant did seem to have a minor positive effect on the activation of piroxicam (3-fold activation), compared to wild-type CYP2C9 (2-fold activation). However, the effect was opposite of what would be expected if the theory of Crespi et al. [6] is followed, which argues that the arginine at position 144 provides an important charge pair for the P450/oxidoreductase interaction. Thus, a decrease in the degree of activation in this variant with piroxicam would have been expected, due to reduced electron flow. This argues that the R144C variant more likely alters piroxicam activation by having the less bulky cysteine residue present in the active site, resulting in enhanced productive binding by piroxicam.

Kinetic studies with dapsone and flurbiprofen support the theory that dapsone and flurbiprofen have their own distinctive binding regions within the active site, as dapsone lowered flurbiprofen K_m in all enzymes (Table 4.1), thus increasing the affinity and not displacing flurbiprofen from the active site. The greatest affect was observed by the F114L mutant, where the K_m for flurbiprofen hydroxylation decreased from 246 to 44 μM , additional evidence suggesting the importance of the *pi*-stacking region to the activation of flurbiprofen. In a previous manuscript, we have measured both flurbiprofen and dapsone metabolite in a single incubation [18], further supporting the presence of

dapsone in the active site, and thus the two-site binding model. In addition, Lineweaver-Burke plots shown in figure 4.3-4.6 were indicative of ternary complex formation, except for the *3 variant (Figure 4.5). Reasons for this may be the almost linear Michaelis-Menten fits due to substantially lower activity. The ternary complex model is in opposition to the ping pong model, which describes the release of one product at a time. It appears likely then that dapsone binding within the CYP2C9 active site causes a change in the active site conformation, resulting in a heterotropic positive cooperativity that differentially affects CYP2C9 substrates, depending upon binding location in the active site.

Overall, results suggest that the interactions important for activation most likely take place in the active site, as the F114L mutant displayed enhanced activation for flurbiprofen, while diminishing activation for piroxicam, and not affecting naproxen activation. This, coupled with naproxen being activated substantially more than flurbiprofen or piroxicam support the notion that the level of CYP2C9 activation by dapsone is substrate dependent. These results provide additional evidence that dapsone activation (positive cooperativity) occurs as a result of binding interactions within the CYP2C9 active site, and not at an external allosteric site. Studies utilizing chimeric 2C9/19 and CYP2C19 mutant enzymes to more specifically determine important regions/residues for binding of the prototypical CYP2C9 substrates, as well as spectral binding studies for flurbiprofen in the absence/presence of dapsone are ongoing and will be reported in the next chapter.

Table 4.1. Activation of CYP2C9 Allelic Variant Enzymes by Dapsone

Kinetic parameters for formation of 4'-hydroxy flurbiprofen in absence and presence of dapsone (Standard Error)

$V_{\max}$ expressed as pmol/min/pmol P450, K_m as μM

Treatment		2C9 WT	2C9*2 (R144C)	2C9*3 (I359L)	2C9 (F114L)
$V_{\max}$	0 Dapsone	13.2 (0.3)	3.3 (0.2)	0.46 (0.1)	5.8 (0.3)
	2 μM Dapsone	14.5 (0.9)	3.7 (0.2)	0.59 (0.1)	5.1 (0.2)
	5 μM Dapsone	13.0 (0.4)	3.5 (0.1)	0.78 (0.05)	5.9 (0.1)
K_m	0 Dapsone	30.6 (1.8)	28.1 (4.8)	81.3 (19.9)	246 (21.1)
	2 μM Dapsone	26.0 (4.6)	17.2 (2.9)	73.8 (22.7)	64.4 (4.7)
	5 μM Dapsone	16.0 (1.5)	10.8 (1.1)	64.5 (8.1)	44.1 (1.5)

Figure 4.1. Structures of Three CYP2C9 Substrates

Structures of the three CYP2C9 substrates studied for activation by dapsone. Arrows indicate site of metabolism.

Figure 4.2. Screen of Dapsone Activation of CYP2C9 Allelic Variants and F114L Mutant

Activation of flurbiprofen 4'-hydroxylation (Panel A), naproxen demethylation (Panel B), and piroxicam 5'-hydroxylation (Panel C) by 10 μ M dapsone in CYP2C9 WT, *2 (R144C) and *3 (I359L) allelic variants, and F114L mutant. Flurbiprofen, naproxen and piroxicam concentrations were also at 10 μ M. Numbers in parentheses above shaded bars indicate (-fold) activation by dapsone.

Figure 4.3. Dapsone Activation of Flurbiprofen 4'-Hydroxylation in CYP2C9 Wild Type

Kinetic effects of 2 and 5 μM dapsone on flurbiprofen 4'-hydroxylation in purified CYP2C9 wild-type enzyme. Inset shows Lineweaver-Burke plot displaying formation of a ternary complex.

Figure 4.4. Dapsone Activation of Flurbiprofen 4'-Hydroxylation in CYP2C9 *2

Kinetic effects of 2 and 5 μM dapsone on flurbiprofen 4'-hydroxylation in purified CYP2C9*2 (R144C) variant. Inset shows Lineweaver-Burke plot displaying formation of a ternary complex.

Figure 4.5. Dapsone Activation of Flurbiprofen 4'-Hydroxylation in CYP2C9 *3

Kinetic effects of 2 and 5 μM dapsone on flurbiprofen 4'-hydroxylation in purified CYP2C9*3 (I359L) variant. Inset shows Lineweaver-Burke plot displaying lack of formation of a ternary complex.

Figure 4.6. Dapsone Activation of Flurbiprofen 4'-Hydroxylation in F114L Mutant

Kinetic effects of 2 and 5 μM dapsone on flurbiprofen 4'-hydroxylation in purified CYP2C9 F114L mutant. Inset shows Lineweaver-Burke plot displaying formation of a ternary complex.

CHAPTER V

Additional Studies into the Mechanism of CYP2C9 Activation

5.1. Introduction

The cytochrome P450 superfamily of enzymes are the most important enzymes responsible for metabolism of exogenous xenobiotic substrates. It has been found that many of these substrates, particularly those metabolized by CYP3A4, display kinetic profiles that are not consistent with the Michaelis-Menten model. In fact, it was initially thought that CYP3A4 was the only major CYP isoform contributing to atypical kinetic profiles. Other groups have since shown that other CYP isoforms can display atypical kinetics as well [1-3]. Recently, our group has shown that dapsone, a compound used to treat *Pneumocystis carinii* infections in AIDS patients, can activate the metabolism of a number of CYP2C9 substrates, including flurbiprofen, naproxen, and piroxicam [4]. Kinetic evidence from this study supports our main hypothesis, which is that both effector (dapsone) and the substrate may be present in the active site simultaneously, both having access to the reactive oxygen. Studies have been conducted to determine the structural requirements for this activation (chapter 3), as well as the effects of amino acid mutations on the ability of dapsone to activate CYP2C9-mediated metabolism (chapter 4). However, evidence is still lacking for what regions or residues within the active site are important in the catalysis of certain CYP2C9 substrates relative to one another within the active site, and just how dapsone affects this binding and/or orientation of substrates.

It has been proposed that the CYP2C enzymes possess six substrate recognition sites (SRSs) or regions that variably recognize and bind substrates [5]. One approach to determining the important regions and residues within a given CYP for catalytic activity is the use of chimeric and mutant enzymes. Human P450s CYP2C9 and CYP2C19 both

metabolize a number of clinically relevant drugs. However, these isoforms have very different substrate specificities despite differing in only 43 of 490 amino acids. For example, CYP2C9 metabolizes many NSAIDs such as flurbiprofen [6;7], naproxen [8;9], and ibuprofen [10], whereas CYP2C19 lacks substantial activity towards these substrates. As a result, amino acid regions may be interchanged between these enzymes through cloning techniques to determine which ones are vital to binding certain substrates or inhibitors. For example, it has been shown that by replacing amino acids 227-338 (SRS 3 and 4) in CYP2C19 with the corresponding region of CYP2C9, a high-affinity enzyme for binding of the CYP2C9 inhibitor sulfaphenazole was created [11]. In addition, it was found that two amino acid substitutions in CYP2C19 (N286S, I289N), which are present in SRS 4, also result in high-affinity sulfaphenazole binding [11]. This same technique was utilized to determine binding regions important for catalytic activity towards flurbiprofen, naproxen, and piroxicam, as well as activation of these chimeric and mutant enzymes by dapsone.

Despite kinetic evidence suggesting the presence of dapsone in the active site during activation of CYP2C9 [4], and literature suggesting that dapsone is also a substrate for CYP2C9 [12], conclusive evidence for the mechanism of how dapsone causes activation is still lacking. Examples of possible mechanisms are that dapsone simply causes flurbiprofen to bind with higher affinity, or in a more productive orientation due to steric or electronic constraints imposed upon the active site. A technique often used to measure affinity for binding to CYP isoforms is difference visible spectroscopy, or spectral binding studies. To date, spectral binding studies involving CYP2C9 are few in number. However, spectral binding studies have been performed to

determine structural requirements for sulfaphenazole inhibition of CYP2C9 [13;14], for which it is a known potent, selective inhibitor [15] [16]. We have performed similar studies with the goal of determining if the presence of dapsone directly affects the affinity of CYP2C9 for binding of flurbiprofen, as kinetic evidence suggests [4].

In addition to dapsone affecting the binding affinity of substrates, it may also affect the binding orientation of substrate within the active site. Ibuprofen is a chiral substrate for CYP2C9, with both the (S)- and (R)-enantiomers being 2- and 3-hydroxylated [10]. Having two major products from both enantiomers has allowed study of the regiospecificity and enantioselectivity for activation of CYP2C9-mediated ibuprofen metabolism. Similar studies have been performed with CYP3A4 by studying the ratio of 1'- and 4-hydroxy triazolam formed from triazolam under different incubation conditions [17]. Thus by observing a difference in the ratio of 2- and 3-hydroxy ibuprofen formation, the effects of dapsone on the relative orientation of ibuprofen within the active site will be studied in hopes of discovering additional evidence for the presence of multiple binding regions within the CYP2C9 active site.

In addition to dapsone analog studies for activation of CYP2C9-mediated flurbiprofen 4'-hydroxylation (chapter 3), it has been theorized that perhaps the major metabolite of dapsone, hydroxy dapsone, could also be an activator of CYP2C9. To our knowledge, this would represent the first example of a non-parent compound (metabolite) activating CYP-mediated metabolism. Consequently, studies were performed to screen the ability of hydroxy-dapsone to activate flurbiprofen, naproxen, and piroxicam metabolism. Overall, this group of studies has been performed to gain an increased

understanding of the binding characteristics and activation of CYP2C9-mediated metabolism.

5.2. Methods

Chemicals. Acetonitrile and dibasic potassium phosphate were obtained from Fisher Scientific Co. (Pittsburgh, PA). (S)-Flurbiprofen, 4'-hydroxy flurbiprofen, and 2-fluoro-4-biphenyl acetic acid (internal standard) were gifts from Pharmacia Corp. (Kalamazoo, MI). Dapsone was purchased from Sigma Chemical Co. (St. Louis, MO) and hydroxy-dapsone was a gift from Reggie Frye at Pittsburgh University. All other chemicals were obtained from commercial sources and were of the highest purity available. CYP2C9/19 chimeric enzymes were constructed by Dr. Robert L. Haining and provided to us by Dr. Allan E. Rettie at the University of Washington as described below. CYP2C19 mutant enzymes were also provided by Dr. Allan E. Rettie at the University of Washington and constructed as described below. CYP2C9 expressed microsomes were a gift from Camitro Corp. (Menlo Park, CA).

Chimera construction. The plasmids pFB2C9 and pFB2C19 were used for all DNA manipulations, taking advantage of the common, unique, and naturally occurring restriction-endonuclease sites SmaI (at the codon specifying amino acid 228), PstI (291-292), and SphI (340) within each cDNA. Standard techniques were used for the digestion, analysis, isolation, and subsequent re-ligation of subcloned DNA fragments. Insertions were confirmed by restriction mapping and the complete re-sequencing of each newly created cDNA. Reconstructed plasmids (pFB2C9/19 [340-490], pFB2C19/9 [340-490], pFB2C19/9 [228-292], pFB2C9/19 [228-292], pFB2C9/19 [228-340]) were used

directly in the creation of recombinant baculovirus DNA by the Bac-to-BacTM method (Life Technologies). Recombinant ‘bacmid’ DNA was then used to transfect T.ni cells, resulting in a long-term viral stock for subsequent amplification and infection of T.ni expression cultures. All five purified chimeric proteins provided typical P450 spectra with little or no evidence of P420 formation upon treatment with carbon monoxide and sodium dithionite.

Chimera incubation conditions. Chimeric P450 enzymes were reconstituted with P450 reductase and cytochrome b5 in a 1:2:1 ratio (5 pmol P450: 10 pmol reductase: 5 pmol b5 per incubation) with dilauroylphosphatidylcholine vesicles (10 µg) extruded through a 200 nm filter. The reconstituted enzymes were incubated in the presence of 300 µM (S)-flurbiprofen, (S)-naproxen, or piroxicam and 50 mM potassium phosphate buffer, pH 7.4 in a total volume of 200 µL (in triplicate). Reactions were initiated by the addition of 1mM NADPH and carried out for 20 minutes (45 minutes for piroxicam) at 37°C. Incubations with flurbiprofen were quenched by adding 200 µl acetonitrile containing internal standard (180 ng/mL 2-fluoro-4-biphenylacetic acid), followed by addition of 40 µl ½-strength H₃PO₄. Incubations with naproxen were quenched by adding 200 µl acetonitrile followed by addition of 40 µl ½-strength H₃PO₄, while piroxicam reactions were quenched by adding 20 µl perchloric acid, followed by directly placing incubation tubes on ice. Samples from all incubations were then centrifuged at 10,000 rpm for 5 minutes, placed into autosampler vials, and 10-100 µl injected onto HPLC system. Incubations examining dapsone (20 µM) activation of chimeras exhibiting flurbiprofen hydroxylase activity were carried out as described above with 300 µM (S)-flurbiprofen.

CYP2C19 mutant enzymes. CYP2C19 mutant enzymes are labeled as 2C19 KS, KSN, KSEN, KN, and SN(2). Amino acid mutations were made at positions 241 (Glu (E) to Lys (K)), 286 (Asn (N) to Ser (S)), 288 (Val (V) to Glu (E)), and 289 (Ile (I) to Asn (N)) in various combinations, and were constructed according to methods previously reported [11]. Incubation conditions of CYP2C19 mutants with (S)-flurbiprofen were same as described above for 2C9/19 chimeric enzymes with regards to activity screening and dapsone activation.

Analysis of 4'-hydroxy flurbiprofen. 4'-Hydroxy flurbiprofen was analyzed by HPLC with fluorescence detection and comparison to a standard dissolved in 50:50 (v/v), acetonitrile:50 mM potassium phosphate buffer pH 7.4. The HPLC system consisted of a Waters® 501 HPLC pump, a Waters® 717 autosampler and a Waters® 470 fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm. The mobile phase consisted of 45:55 (v/v), acetonitrile:20 mM K₂HPO₄, pH 3.0 pumped at 1 ml/min through a Brownlee Spheri-5® C₁₈ 4.6 x 100 mm column. 4'-Hydroxy flurbiprofen and internal standard eluted at approximately 2.2 and 4.8 minutes, respectively.

Analysis of desmethylnaproxen. Desmethylnaproxen was analyzed identically to 4'-hydroxy flurbiprofen except that the fluorescence detector was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm, and no internal standard was used. Desmethylnaproxen eluted at approximately 2.1 minutes.

Analysis of 5'-hydroxy piroxicam. 5'-Hydroxy piroxicam was analyzed by HPLC with UV detection (365 nm). The HPLC system was as described above, but was fitted to a Waters® 486 UV detector. The mobile phase consisted of 50:50 (v/v), acetonitrile:50

mM K₂HPO₄, pH 3.0 pumped at 1 mL/min through a Phenomenex Luna C₁₈ 5 µm, 4.6 x 150 mm column. Retention time for 5'-hydroxy piroxicam was approximately 4.3 minutes.

Data Presentation. All bar graph data of CYP2C9/19 chimeric and 2C19 mutant enzymes are presented as mean of three determinations with error bar signifying standard deviation.

Hydroxy-dapsone activation of flurbiprofen. Hydroxy dapsone was incubated with (S)-flurbiprofen (2-300µM) in a 6x6 matrix design experiment containing 1 pmol expressed CYP2C9 microsomes co-expressed with reductase (Camitro Corporation, Menlo Park, CA). Hydroxy dapsone was dissolved in methanol and added to incubations such that incubations did not exceed 2.5% organic concentration, with appropriate controls used. Final incubation volume was 0.2 ml and reactions were initiated with 1 mM NADPH and allowed to incubate at 37°C for 20 minutes. Incubations were quenched by adding 200 µl acetonitrile containing internal standard (180 ng/mL 2-fluoro-4-biphenylacetic acid), followed by addition of 40 µl ½-strength H₃PO₄. Samples were then centrifuged at 10,000 rpm for 5 minutes, placed into autosampler vials, and 30-50 µl injected onto the HPLC system. 4'-Hydroxy flurbiprofen was analyzed as described above, and data were fit in Sigma Plot (Version 6.0) to the two-site model equation [1]:

$$v = \frac{V_{\max} \bullet [S]}{K_m \left(\frac{1 + \frac{[B]}{K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right) + [S] \left(\frac{1 + \frac{[B]}{\alpha K_B}}{1 + \frac{\beta[B]}{\alpha K_B}} \right)} \quad (5.1)$$

where S is the substrate, B is the effector, $V_{\max}$ and K_m are kinetic constants for substrate metabolism, K_B is the binding constant for the effector, α is the change in K_m resulting from effector binding, and β is the change in $V_{\max}$ from effector binding. For activation, $\alpha < 1$ and/or $\beta > 1$.

Spectral Binding Studies. Expressed CYP2C9 microsomes (gift from Camitro Corp.) were diluted from 0.7 to 0.2 μM in 50mM potassium phosphate buffer with 1mM EDTA and 1mM N-acetyl cysteine (an antioxidant to prevent breakdown of hydroxy-dapsone), at pH 7.4, and equally divided between sample and reference blocked cuvettes (900 μL). The instrument used was a DW-2000 UV-Vis Spectrophotometer (SLM Instruments, Inc.), specifically designed to analyze turbid samples. In the study with flurbiprofen alone, 5 μL of a methanolic solution (6% DMSO) was added to both cuvettes as a background control, since both dapsone and hydroxy-dapsone were dissolved in this vehicle. After a baseline was recorded, 5 μL aliquots of racemic-flurbiprofen was added to the sample cuvette, with the same volume of 100mM potassium phosphate pH 7.4 being added to the reference cuvette. After 3 minutes, the difference spectra were recorded between 350 and 500nm at room temperature. Concentrations of flurbiprofen ranged to 155 μM with successive aliquot additions. The binding experiments of flurbiprofen in the presence of dapsone and hydroxy dapsone (20 μM added at the beginning) were carried out exactly the same as flurbiprofen alone. The slit width was set to 6.0nm and a scan rate of 1.0nm/sec was used. The difference in absorbance between the peak (~390nm) and trough (~420nm) of a type I spectra was then plotted vs. flurbiprofen concentration and analyzed by nonlinear regression (Sigma Plot, Version

6.0) to estimate the K_s (spectral binding constant) from the titration binding curve using the following equation:

$$\Delta A = B_{\max}S/K_s + S \quad (5.2)$$

Ibuprofen-Dapsone Incubation Conditions. Human liver tissue for preparation of microsomes was obtained through the Liver Tissue Procurement and Distribution System. Based on chart review, none of the patients from whom tissues were obtained was taking any medications known to inhibit or induce the P450s. Microsomal samples were prepared according to established methods [18]. Protein content was measured by the method of Lowry et al. [19] and P450 content by the method of Omura and Sato [20]. The final microsomal protein concentration was 200 $\mu\text{g/mL}$ and the total incubation volume was 500 μL in 100 mM potassium phosphate, pH 7.4. Either (R)- or (S)-ibuprofen (5-200 μM) was added to each tube in the absence or presence of 5 or 10 μM dapsone, and reactions were initiated with 1 mM NADPH and allowed to run for 20 minutes at 37°C. Reactions were quenched by addition of 500 μL of ethyl acetate:hexane (80:20 v/v), and 200 ng ketoprofen was added as internal standard, followed by addition of 50 μL 40% phosphoric acid. Next, 3 mL ethyl acetate:hexane (80:20 v/v) was added and samples were vortexed for 20 seconds, then centrifuged at 2000 g for 10 minutes. The organic layer was then pipetted off and transferred to a 13x100 tube and evaporated to dryness. These tubes were then sent to Mitch Hamman at Indiana University Division of Clinical Pharmacology for analysis. Data were analyzed and fit the Michaelis-Menten equation to generate K_m and $V_{\max}$ estimates for ibuprofen 2- and 3-hydroxylation.

$$v = \frac{V_m \bullet S}{K_m + S} \quad (5.3)$$

5.3. Results

CYP2C9/19 Chimeric Enzyme Studies. CYP2C9/19 chimeric enzymes (Figure 5.1) were constructed in order to determine which amino acid regions were important for catalysis of flurbiprofen, naproxen, and piroxicam. This provided insight into the active site binding characteristics of these compounds. Results from incubations with CYP2C9/19 chimeric enzymes and flurbiprofen show that chimeras 2C9/19 (340-490) and 2C19/9 (228-292) had flurbiprofen hydroxylase activity, with mean velocities of 7.38 and 6.92 pmol/min/pmol P450, respectively (Figure 5.2). All other chimeras exhibited minimal activity towards flurbiprofen. These same chimeras catalyzed naproxen demethylation, although less than CYP2C9 wild-type (Figure 5.3), while only the 2C19/9 (228-292) chimera exhibited substantial piroxicam hydroxylase activity (Figure 5.4). In fact, this chimera showed higher piroxicam hydroxylase activity than wild-type CYP2C9 (Figure 5.4). For activation studies with dapsone involving the chimeras with flurbiprofen hydroxylase activity, the 2C9/19 (340-490) chimera was activated in the presence of 20 μ M dapsone 1.2-fold, while the 2C19/9 (228-292) chimera showed no activation (Figure 5.5).

CYP2C19 Mutant Enzyme Studies. CYP2C19 mutant enzymes were constructed to determine which residues could confer flurbiprofen hydroxylase activity to CYP2C19, which has minimal activity towards this substrate. As can be seen in Figure 5.6, the 2C19 KSN and KSEN mutants had the greatest activity towards flurbiprofen, with the

KSN mutant exhibiting higher activity than wild-type CYP2C9. Activation studies with these enzymes showed that dapsone did not activate flurbiprofen hydroxylase activity in either of these CYP2C19 mutants (Figure 5.5).

Hydroxy-Dapsone Activation of Flurbiprofen 4'-Hydroxylation. Hydroxy-dapsone (HDA), the major phase I metabolite of dapsone, was found to activate flurbiprofen 4'-hydroxylation. Data were fit to the two-site effector model equation (equation 5.1) to generate a surface plot (Figure 5.7), yielding an α value of 0.98 and a β of 1.5 (Table 5.1), indicating no effect on K_m , but a increase in V_{max} . This is different from dapsone, which affected both the K_m and V_{max} of flurbiprofen 4'-hydroxylation (Figure 5.8) with an α of 0.23 and a β of 1.9 (Table 5.1). Eadie Hofstee plots were prepared to confirm effects on K_m and V_{max} of flurbiprofen 4'-hydroxylation by hydroxy-dapsone (Figure 5.9) and dapsone (Figure 5.10). Interestingly, activation by hydroxy-dapsone became saturated around 50 μ M, while activation by dapsone saturated around 100 μ M (Figures 5.7 and 5.8).

Spectral Binding Studies. To determine whether dapsone and hydroxy-dapsone affect binding of flurbiprofen directly, spectral binding titration studies were performed. Plotting the difference in absorbance between the peak at ~390 nm and trough at ~420 nm vs. flurbiprofen concentration yields a hyperbolic curve that can be fit by nonlinear regression to equation 5.2 to estimate the spectral binding constant (K_s). In the absence of effector, flurbiprofen gave a type I spectra (Figure 5.11, A) with a K_s of 14.1 μ M (Figure 5.11, B), while in the presence of 20 μ M dapsone, flurbiprofen also gave a type I spectra (Figure 5.12, A), but with a lower K_s of 2.1 μ M (Figure 5.12, B), indicating that the presence of dapsone increases the affinity of CYP2C9 for flurbiprofen. On the other

hand, in the presence of hydroxy-dapsone, flurbiprofen gave a type I spectra (Figure 5.13, A), with a K_s of 14.0 μM (Figure 5.13, B), similar to flurbiprofen alone, suggesting that hydroxy-dapsone does not affect binding of flurbiprofen directly, agreeing with kinetic data showing no effect of hydroxy-dapsone on the K_m of flurbiprofen.

Ibuprofen-Dapsone Interaction. In the absence of dapsone, ibuprofen is regioselectively metabolized, with 3-hydroxylation being greater than 2-hydroxylation (Figure 5.14). In the presence of dapsone, however, 3-hydroxylation of (S)-ibuprofen was substantially activated (Figure 5.15A), with the $V_{\max}$ increasing from 1435 to 3026 pmol/min/mg protein and K_m decreasing from 14.2 to 9.2 μM (Table 5.2). Meanwhile, dapsone minimally affected 2-hydroxylation of (S)-ibuprofen (Figure 5.15B). In contrast, dapsone inhibited both the 3- and 2-hydroxylation of (R)-ibuprofen (Figures 5.16A and B), as $V_{\max}$ values decreased from 408 to 281 pmol/min/mg protein and 765 to 569 pmol/min/mg protein, respectively (Table 5.2).

5.4. Discussion

In this study, CYP2C9 substrate metabolism was monitored by using CYP2C9/19 chimeric and CYP2C19 mutant enzymes to better understand important binding regions/residues within the active site, and perhaps shed some light on atypical kinetics (i.e. activation) observed by this isoform. In addition, the direct effect of dapsone, as well as hydroxy-dapsone, on binding of flurbiprofen was studied to provide mechanistic information of why dapsone activates flurbiprofen 4'-hydroxylation, and to confirm observed kinetic effects of dapsone on CYP2C9. Lastly, the effect of dapsone on the regioselectivity of ibuprofen metabolism was studied to show how the presence of

effector can potentially alter the binding orientation of substrate within the active site of CYP2C9. Briefly, the CYP2C19/9 (228-292) and 2C9/19 (340-490) chimeras displayed good activity towards flurbiprofen, naproxen, and piroxicam, while the CYP2C19 KSN mutant showed the greatest activity towards flurbiprofen, suggesting the importance of residues 241, 286, and 289 in catalysis of this substrate. Dapsone was only able to activate flurbiprofen 4'-hydroxylation in the 2C9/19 (340-490) chimera. It was also discovered that hydroxy-dapsone can activate flurbiprofen 4'-hydroxylation similar to dapsone, although primarily through a $V_{\max}$ effect, which spectral binding studies confirmed. Lastly, ibuprofen metabolism was regioselectively and stereoselectively altered by dapsone, suggesting a change in ibuprofen binding orientation in the presence of dapsone.

Despite the numerous studies describing atypical kinetics such as positive cooperativity, progress has been limited in clearly defining the underlying causes of this phenomenon. Cytochrome P450 3A4 is particularly interesting, considering it is the most abundant CYP enzyme and metabolizes numerous compounds [21;22]. It has been observed that many substrates of CYP3A4 show atypical kinetic profiles [1;23-26], and thus, with respect to atypical kinetics, CYP3A4 has been by far the most studied isoform. The preponderance of evidence suggests that there are at least two distinct binding regions within the CYP3A4 active site. One model in particular suggests that both substrate and effector may be present in the active site simultaneously, each having access to the reactive oxygen [1;24]. Our lab has shown evidence that this two-site model applies to CYP2C9, an isoform for which dapsone has been shown to cause heterotropic positive cooperativity (activation) [4]. However, this study only provides

kinetic evidence to support the simultaneous presence of multiple compounds within the active site. Studies exploring binding within the active site would be more helpful in understanding the interactions necessary for positive cooperativity to occur.

Studies with CYP2C9/19 chimeric enzymes suggest that amino acid region 228-292 is necessary for catalysis of flurbiprofen, naproxen, and piroxicam, as substantial activity towards these substrates was conferred when this amino acid region from CYP2C9 was placed into CYP2C19. According to Gotoh, this region lies in SRS 3 and part of SRS 4, two of the six proposed substrate recognition sites of CYP2C enzymes [5]. Interestingly, the CYP2C19 mutants KSN and KSEN, which have single mutations within SRS 3 also had good activity towards flurbiprofen 4'-hydroxylation. The mutation at residue 241 (Glutamate to Lysine) is proposed to be within helix G, while those at 286 (Asparagine to Serine), 288 (Valine to Glutamate), and 289 (Isoleucine to Asparagine) are proposed to be within helix I, which is associated with the substrate binding pocket and is one of the most structurally conserved regions of CYP450s [27;28]. Similar studies have been performed to determine important binding regions for other compounds with CYP2C9. Jung et al. reported that replacement of amino acids 227-338 in 2C19 with the corresponding region of 2C9 lead to an enzyme with a high affinity for sulfaphenazole [11]. In addition, two amino acid substitutions in 2C19 (N286S and I289N) conferred high-affinity sulfaphenazole binding [11]. Likewise, Klose et al. found that residues 286 and 289 were important in conferring diclofenac and ibuprofen hydroxylase activity to CYP2C19 [29]. However, it must be understood that this does not necessarily suggest that residues 286 and 289 directly contact the substrate. In fact, based on a homology model generated by Jung et al. [11], these residues are too far away

from the heme to directly contact the substrate. In addition, it was deduced from this homology model that the I289N difference lies at the interface of helix I with helix G, which has a considerable range of motion according to molecular dynamics studies. This mutation could thus serve to alter the motion of these helices, which in turn could affect access of substrate to the heme oxygen or overall protein conformation [11]. However, other factors are likely contributing. Overall, the regions/residues important for catalysis of flurbiprofen, naproxen, and piroxicam appear to coincide with a number of other studies.

The effect of dapsone on chimeric and mutant enzyme activity was also examined. It was found that only the CYP2C9/19 (340-490) chimera could be activated by dapsone, albeit only 1.2-fold. We suspect that this may result from this chimeric enzyme being primarily CYP2C9 (only residues 340-490 are from CYP2C19), thus still possessing the region allowing dapsone to bind and cause positive cooperativity. In addition, the 2C19/9 (228-292) chimera also has residues 340-490 from 2C19, in addition to residues corresponding to SRS 1 and 2, and was unable to be activated by dapsone. On the other hand, SRS 1 and 2 in the 2C9/19 (340-490) chimera (activated) were from CYP2C9, suggesting that these regions may be important for binding dapsone. This is assuming that the catalytic site and effector site for dapsone are one in the same. Interestingly, He et al. [30] suggest that perhaps dapsone may interact electrostatically in the active site through its sulfone group. In addition, Ridderstrom et al. have noted that Arginines 97 and 108 are important in the catalytic activity of CYP2C9 [31], which are present in SRS 1 according to Gotoh [5], implicating this as a possible binding determinant for dapsone. Unfortunately, no chimeric or mutant enzyme has been

constructed to explore this potential binding region and its effects on activation. Until more mammalian P450s are crystallized for modeling these interactions, predictions of binding sites will be difficult and remain theoretical.

In addition to dapsone activating CYP2C9-mediated metabolism, it has also been shown to be a substrate for this isoform [12;32], with N-hydroxy dapsone being the major metabolite. In previous studies, we have explored effector structural requirements for activation of CYP2C9. One compound that we did not initially screen for activation was the hydroxy metabolite of dapsone. Our studies show that hydroxy-dapsone does activate flurbiprofen 4'-hydroxylation, but not naproxen or piroxicam metabolism. To our knowledge, this is the first example of a metabolite that activates CYP-mediated metabolism. Compared to dapsone, however, hydroxy-dapsone causes an increase in $V_{\max}$ without much effect on the K_m for flurbiprofen 4'-hydroxylation, whereas dapsone increases $V_{\max}$ and decreases K_m , as can be seen by α and β values in table 5.1. Eadie-Hofstee plots confirm these effects on K_m and $V_{\max}$ estimates by hydroxydapsone and dapsone (Figures 5.9 and 5.10). In particular, it can be seen that the slope of the Eadie-Hofstee plots in the presence of increasing concentrations of hydroxy-dapsone are relatively parallel, suggesting minimal changes on K_m , whereas slopes in the presence of dapsone are not parallel, indicating a K_m effect. Based on this kinetic evidence, dapsone increases the affinity (decreases K_m) of CYP2C9 for flurbiprofen, whereas hydroxy-dapsone does not. With regard to the $V_{\max}$ effects of hydroxy-dapsone on CYP2C9, similar results have been observed for CYP3A4, as Shou et al. [24] observed that 7,8-benzoflavone increased the $V_{\max}$ for phenanthrene metabolism with minimal changes to the K_m .

To confirm the effects of dapsone and hydroxy-dapsone on binding affinity of flurbiprofen, spectral titration binding studies were performed. Results confirm that the presence of dapsone does cause flurbiprofen to bind CYP2C9 more efficiently, as spectral binding constant (K_s) estimates for flurbiprofen were decreased from 14.1 to 2.1 μM . This increased binding efficiency likely contributes to an increase in catalytic efficiency for CYP2C9-mediated flurbiprofen 4'-hydroxylation. It is conceivable to suggest that the presence of dapsone effectively reduces the size of the active site, thus forcing flurbiprofen to bind more tightly, explaining the K_m and K_s effects observed. On the other hand, the presence of hydroxy-dapsone had no direct effect on binding of flurbiprofen, with K_s values remaining unchanged. Nonetheless, hydroxy-dapsone is still able to activate flurbiprofen 4'-hydroxylation. Reasons for this difference are likely numerous and complicated. One possibility is that hydroxy-dapsone may in some way provide an environment which leads to more efficient electron flow, and hence, reactive oxygen. It has also been suggested that hydrogen bonding and disordering the orientation of water molecules within the active site may affect catalytic activity of the enzyme. For example, through site-directed mutagenesis studies, Atkins and Sligar have found an important role for hydrogen bonding within the active site of cytochrome P450cam [33]. They determined that a hydrogen bond between Tyr96 and the ketone group of camphor placed camphor in such a position as to promote efficient catalysis. By mutating this residue to a phenylalanine (resulting in no H-bond), it was found that the free energy (ΔG) for binding was unaffected, while the ΔG for spin state conversion was much higher compared to wild-type enzyme, with only 59% conversion from low to high spin, versus 95% for wild-type enzyme [33]. This was rationalized by suggesting that the hydrogen

bond placed camphor in such a way as to protect the heme from solvent interaction, thus promoting efficient electron flow to the heme, and hence higher spin state conversion [33]. Other groups have proposed that $V_{\max}$ effects may stem from expulsion of water molecules from the active site, which may help prevent uncoupling of the P450 cycle, leading to production of hydrogen peroxide or excess water formation [34-36]. It is reasonable to suggest that hydroxy-dapsone, although it has a very similar structure, interacts within the active site differently than does dapsone, perhaps disrupting the water orientation and driving the enzyme towards a higher percentage of high-spin state without affecting binding of the activated substrate.

Similar to the NSAIDs flurbiprofen and naproxen, ibuprofen is a substrate whose metabolism is activated by dapsone. Ibuprofen is a chiral substrate for CYP2C9 that undergoes both 2- and 3-hydroxylation for both enantiomers. We have observed that 3-hydroxylation of these enantiomers is greater than 2-hydroxylation, which agrees with a previous study [10]. Interestingly, in the presence of dapsone, the 3-hydroxylation of (S)-ibuprofen is activated (Figure 5.15), while 2-hydroxylation is minimally affected. Meanwhile, dapsone appears to weakly inhibit the 2- and 3-hydroxylation of (R)-ibuprofen. Thus, there appears to be a regioselective, as well as an enantioselective effect of dapsone on ibuprofen metabolism. Results from (S)-ibuprofen suggest that dapsone not only alters the regioselectivity towards 3-hydroxylation, but activates the enzyme overall, as the sum of the velocities for 2- and 3-hydroxylation are substantially increased. This information suggests that dapsone does alter the shape of the active site, such that the binding orientation of ibuprofen is altered, as opposed to just increasing the catalytic activity of the CYP2C9 enzyme through some other mechanism. This is not to

say that alteration of active site shape is the only factor involved, but it likely contributes to activation. Similar studies have been conducted with CYP3A4 and triazolam metabolism. Schrag and Wienkers [17] studied the 1'- and 4-hydroxylation of triazolam by CYP3A4 and observed altered ratios of the 4-hydroxy to 1'-hydroxy metabolite under different incubation conditions. They rationalized their findings by suggesting that data was consistent with a two-site model for binding. Whether the change in active site topology induced by dapsone is a result of dapsone being in the active site, or due to a change in protein conformation still remains unclear. However, kinetic evidence from one of our previous studies suggests the presence of dapsone in the active site simultaneously with substrate [4]. Results for (R)-ibuprofen are different in that dapsone appears to inhibit 2- and 3-hydroxylation of this enantiomer, although rather weakly. It is likely that the (R)-enantiomer itself has a slightly different binding orientation than the (S)-enantiomer, resulting in the observed differences and suggesting multiple binding domains or sites.

Overall, results from these studies provide additional information about binding of substrates to CYP2C9 active site, as well as possible mechanisms of activation, one of which is alteration of binding orientation by dapsone. Perhaps the most interesting result from these studies is the discovery that the major metabolite of dapsone (hydroxy-dapsone) can also serve to activate flurbiprofen 4'-hydroxylation, although apparently through a slightly different mechanism. Future studies will include measuring oxygen consumption and hydrogen peroxide formation by CYP2C9 in the presence of dapsone and hydroxy-dapsone to characterize potential effects on uncoupling mechanisms.

5.5. Acknowledgments

We would like to gratefully acknowledge Dr. Allan Rettie at the University of Washington (Seattle) for providing us with the CYP2C9/19 chimeric and CYP2C19 mutant enzymes. We would also like to thank Dr. Andrew Shiemke from the Biochemistry Department at West Virginia University for the use of his spectrophotometer for the CYP2C9 spectral binding studies.

Figure 5.1. Constructed CYP2C9/19 Chimeric Enzymes

CYP2C9/19 chimeric enzymes constructed for binding region studies. SRS refers to substrate recognition sequences and restriction enzymes are shown at splice sites. All chimeras were composed of 490 residues.

Figure 5.2. Flurbiprofen 4'-Hydroxylation by CYP2C9/19 Chimeric Enzymes

Flurbiprofen 4'-hydroxylation in CYP2C9/19 chimeric enzymes. Activity for CYP2C9 and 2C19 wild-type are included for comparison.

Figure 5.3. Naproxen Demethylation by CYP2C9/19 Chimeric Enzymes

Naproxen demethylation in CYP2C9/19 chimeric enzymes. Activity for CYP2C9 and 2C19 wild-type are included for comparison.

Figure 5.4. Piroxicam 5'-Hydroxylation by CYP2C9/19 Chimeric Enzymes

Piroxicam 5'-hydroxylation in CYP2C9/19 chimeric enzymes. Activity for CYP2C9 and 2C19 wild-type are included for comparison.

Figure 5.5. Dapsone Activation in CYP2C9/19 Chimeric and 2C19 Mutant Enzymes

Screen for activation of flurbiprofen 4'-hydroxylation in active 2C9/19 chimeras and 2C19 mutants.

Figure 5.6. Flurbiprofen 4'-Hydroxylation in CYP2C19 Mutant Enzymes

Flurbiprofen 4'-hydroxylation in E.coli purified CYP2C19 mutant enzymes. Mutated residues are explained in the Methods section.

Figure 5.7. Hydroxy-Dapsone Activation of Flurbiprofen 4'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of flurbiprofen 4'-hydroxylation by hydroxy-dapsone. The surface is the fit to equation 5.1.

Figure 5.8. Dapsone Activation of Flurbiprofen 4'-Hydroxylation (3-D Plot)

Three-dimensional surface plot showing activation of flurbiprofen 4'-hydroxylation by dapsone. The surface is the fit to equation 5.1.

Table 5.1. Activation of Flurbiprofen 4'-Hydroxylation by Hydroxy-Dapsone and Dapsone

Kinetic parameters for activation of flurbiprofen 4'-hydroxylation by dapsone and hydroxy-dapsone (HDA). Parameters were estimated by fitting data to equation 5.1.

Effector	K_m (SE) (μ M)	V_{max} (SE) (pmol/min/pmol P450)	K_B (SE) (μ M)	α (SE)	β (SE)	R^2
Dapsone	6.6 (0.5)	6.8 (0.2)	35.7 (5.7)	0.2 (0.03)	1.9 (0.06)	0.99
HDA	9.5 (1.2)	7.3 (0.3)	2.6 (0.8)	0.98 (0.2)	1.5 (0.1)	0.99

K_m is the Michaelis-Menten constant; V_{max} is the maximal velocity; K_B is the binding constant for the effector; α is the change in K_m resulting from effector binding; β is the change in V_{max} resulting from effector binding. An α or β value=1 indicates no change in K_m or V_{max} , respectively.

Figure 5.9. Eadie-Hofstee Plot for Hydroxy-Dapsone Effect on Flurbiprofen 4'-Hydroxylation

Eadie-Hofstee plots showing the effect of hydroxy-dapsone on flurbiprofen 4'-hydroxylation. Relatively parallel lines indicates minimal effect on K_m , while different y-axis intercepts indicate increase in V_{max} .

Figure 5.10. Eadie-Hofstee Plot for Dapsone Effect on Flurbiprofen 4'-Hydroxylation

Eadie-Hofstee plots showing the effect of dapsone on flurbiprofen 4'-hydroxylation. Different slopes indicate effect on K_m while different y-axis intercepts indicate increase in V_{max} .

Figure 5.11. Flurbiprofen Type I Difference Spectra

Titration of CYP2C9 with flurbiprofen alone. (A) Observed type I difference spectra. (B) Plot of ΔA 390-420 (from A) vs flurbiprofen concentration fit to equation 5.2 with a $K_s = 14.1$.

A

B

Figure 5.12. Flurbiprofen Type I Difference Spectra in Presence of Dapsone

Titration of CYP2C9 with flurbiprofen in presence of 20 μM dapsone. (A) Observed type I difference spectra. (B) Plot of ΔA 390-420 (from A) vs flurbiprofen concentration fit to equation 5.2 with a $K_s = 2.1$.

Figure 5.13. Flurbiprofen Type I Difference Spectra in Presence of Hydroxy-Dapsone

Titration of CYP2C9 with flurbiprofen in the presence of 20 μM hydroxy-dapsone. (A) Observed type I difference spectra. (B) Plot of ΔA 390-420 (from A) vs flurbiprofen concentration fit to equation 5.2 with a $K_s = 14.0$.

Figure 5.14. Ibuprofen Metabolism

Metabolic pathways for both (S)- and (R)-ibuprofen.

Figure 5.15. (S)-Ibuprofen 2- and 3-Hydroxylation

(S)-ibuprofen 3-hydroxylation (A) and 2-hydroxylation (B) in absence and presence of 5 and 10 μM dapsone. Fits were to equation 5.3.

Figure 5.16. (R)-Ibuprofen 2- and 3- Hydroxylation

(R)-ibuprofen 3-hydroxylation (A) and 2-hydroxylation (B) in the absence and presence of 5 and 10 μM dapsone. Fits were to equation 5.3.

Table 5.2. Effect of Dapsone on (S)- and (R)-Ibuprofen Metabolism

Kinetic parameters for (S)- and (R)-ibuprofen metabolism in the absence and presence of dapsone. Data were fit to Michaelis-Menten equation (equation 5.3).

(S)-Ibuprofen 3-hydroxylation			(S)-Ibuprofen 2-hydroxylation	
Dapsone	$V_{\max}$ (SE)	K_m (SE)	$V_{\max}$ (SE)	K_m (SE)
μM	<i>pmol/min/mg protein</i>	μM	<i>pmol/min/mg protein</i>	μM
0	1435.1 (89.7)	14.2 (4.0)	615.4 (40.3)	18.5 (5.1)
5	1949.5 (139.2)	12.4 (4.2)	472.1 (64.6)	66.2 (23.7)
10	3025.2 (117.2)	9.2 (1.9)	700.8 (43.5)	26.9 (6.1)

(R)-Ibuprofen 3-hydroxylation			(R)-Ibuprofen 2-hydroxylation	
Dapsone	$V_{\max}$ (SE)	K_m (SE)	$V_{\max}$ (SE)	K_m (SE)
μM	<i>pmol/min/mg protein</i>	μM	<i>pmol/min/mg protein</i>	μM
0	764.3 (65.2)	23.7 (7.7)	407.8 (41.3)	19.6 (8.1)
5	749.1 (81.8)	13.1 (6.6)	313.8 (17.4)	11.4 (3.1)
10	569.4 (65.6)	16.6 (8.3)	281.0 (30.3)	14.4 (7.0)

CHAPTER VI

Sensitive and Specific High-Performance Liquid Chromatographic Assay for 4'-Hydroxyflurbiprofen and Flurbiprofen in Human Urine and Plasma

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6.1. Introduction

Flurbiprofen is an arylpropionic non-steroidal anti-inflammatory drug used in the treatment of pain and inflammation. In humans, flurbiprofen is eliminated primarily by oxidation to form 4'-hydroxyflurbiprofen (Fig. 6.1), which accounts for about 86% of the oxidative metabolites [1]. The oxidative metabolites and parent compound are also subject to glucuronidation or sulfation [2;3]. It has been shown that the formation of 4'-hydroxyflurbiprofen is mediated almost exclusively by cytochrome P450 2C9 in a non-stereoselective manner [4;5]. As a result, flurbiprofen may be used as an *in vitro* probe for CYP2C9 activity, without the need for measuring the enantiomers. More recently, it has been shown that flurbiprofen may be used as an *in vivo* probe to estimate the activity of CYP2C9 in human subjects [6].

Other HPLC methods have been described for the detection of racemic flurbiprofen and its metabolites in urine and plasma [3;7-9], in which the analytes must be extracted from their respective biological matrices. This paper describes a sensitive and specific procedure for the detection of flurbiprofen and its 4'-hydroxy metabolite in which no extraction step is utilized, thus reducing the amount of time and error involved in quantitation. The method involves a reverse-phase liquid chromatography column and fluorescence detection, and is sufficiently sensitive to quantitate flurbiprofen and 4'-hydroxyflurbiprofen in amounts as low as 0.25 µg/ml and 0.05 µg/ml, respectively. This assay was used to quantitate concentrations of flurbiprofen and 4'-hydroxyflurbiprofen in urine and plasma from human subjects to evaluate the use of flurbiprofen as an *in vivo* probe for cytochrome P450 2C9 activity.

6.2. Methods

Reagents and chemicals. Acetonitrile, dibasic potassium phosphate buffer, hydrochloric acid and phosphoric acid were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA). *rac*-Flurbiprofen was purchased from Sigma Chemical (St. Louis, MO, USA). 4'-Hydroxyflurbiprofen and 2-fluoro-4-biphenylacetic acid (internal standard (IS)) were gifts from Pharmacia (Kalamazoo, MI, USA). All other chemicals were obtained from commercial sources and were of the highest purity available. Drug-free human plasma was obtained from the blood bank and drug-free human urine was obtained from laboratory personnel.

Instrumentation. The HPLC system consisted of a Waters 501[®] HPLC pump, a Waters 717[®] autosampler and a Waters 470[®] scanning fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm (Waters Assoc., Milford, MA, USA). The mobile phase for both urine and plasma consisted of acetonitrile—20 mM K₂HPO₄, pH 3.0 (40:60, v/v) pumped at 1 ml/min through a Brownlee Spheri-5[®] C₁₈ 4.6 x 100 mm reversed phase column, with a typical pressure of 67 bar.

Preparation of stock solutions. Flurbiprofen and 4'-hydroxyflurbiprofen stock solutions were prepared by dissolving each in 50:50 acetonitrile:H₂O to make a 1 mg/ml solution. From this stock solution, dilutions were made to prepare 50, 10, 5, and 0.5 µg/ml solutions for preparation of the standards. 2-fluoro-4-biphenylacetic acid (IS) was dissolved in acetonitrile to prepare a 1 mg/ml stock solution. From this stock solution a 1000 ng/ml solution was prepared by adding 0.5 ml of the 1 mg/ml stock to 500 ml

acetonitrile. A 500 ng/ml internal standard solution was prepared by adding 0.25 ml of the 1 mg/ml stock to 500 ml acetonitrile. All stock solutions were stored at 4°C.

Flurbiprofen and 4'-hydroxyflurbiprofen in urine. Urine samples (50 µl) were prepared by adding 400 µl of purified water and 100 µl 6 M HCl, followed by vortexing and incubation at 90°C for 30 minutes to facilitate glucuronide cleavage via acid-hydrolysis. Following incubation, 500 µl acetonitrile containing IS (1000 ng/ml of 2-fluoro-4-biphenylacetic acid) was added and the samples vortexed. The samples were then centrifuged at 14,000g for 5 minutes, and 150 µl of sample transferred to autosampler vials and 3-12 µl injected onto the HPLC system for analysis. Standard amounts for flurbiprofen and 4'-hydroxyflurbiprofen ranged from 0.5 to 40 µg/ml (0, 0.5, 1.0, 2.0, 5, 10, 20, and 40 µg/ml). Appropriate amounts of 4'-hydroxyflurbiprofen and flurbiprofen were added to 50 µl blank urine to prepare standards and then brought to final volume of 450 µl by addition of purified water and treated as subject samples.

Flurbiprofen and 4'-hydroxyflurbiprofen in plasma. Plasma samples (100 µl) were prepared by adding 100 µl acetonitrile, 200 µl IS (500 ng/ml of 2-fluoro-4-biphenylacetic acid in acetonitrile), and 40 µl of ½-strength H₃PO₄ (50:50 H₃PO₄ : H₂O), followed by vortexing. The samples were then centrifuged at 14,000g for 10 minutes, and 150 µl placed into autosampler vials and 20-75 µl injected onto the HPLC. The standards were prepared to contain concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0 µg/ml for 4'-hydroxyflurbiprofen and 0, 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 15 µg/ml for flurbiprofen. Appropriate amounts of 4'-hydroxyflurbiprofen and flurbiprofen were added to 100 µl blank plasma and acetonitrile was then added to bring the final volume to 200 µl, followed by treatment as with the subject samples.

Assay validation procedure. Urine assay validation consisted of replicates of a low concentration (1 µg/ml), a middle concentration (12 µg/ml), and a high concentration (30 µg/ml) quality control (QC) sample for flurbiprofen and 4'-hydroxyflurbiprofen. Plasma assay validation QC concentrations were 1 µg/ml, 12 µg/ml, and 30 µg/ml for flurbiprofen, and 0.1 µg/ml, 0.5 µg/ml, and 1.5 µg/ml for 4'-hydroxyflurbiprofen. Intra-day variability was tested with 12 replicates of each quality control concentration. Inter-day variability was conducted over three days with 6 replicates of each QC concentration. Furthermore, low and high QC concentrations (in triplicate) were subjected to three freeze-thaw cycles and analyzed. Finally, processed stability after pretreatment was tested by injecting low and high QC samples every 2 hours for 24 hours. Means, standard deviations and coefficients of variation were calculated by standard methods.

6.3. Results

Representative HPLC chromatograms illustrating the analysis of 4'-hydroxyflurbiprofen and flurbiprofen in urine and plasma, as well as a blank for each, are shown in Figures 6.2-6.5. 4'-Hydroxyflurbiprofen, IS, and flurbiprofen were separated within 16 minutes, with retention times of approximately 3.6, 9.0, and 14.0 minutes, respectively. The standard curves for 4'-hydroxyflurbiprofen and flurbiprofen in urine were linear through 40 µg/ml, while the curves for 4'-hydroxyflurbiprofen and flurbiprofen in plasma were linear through 3 µg/ml and 15 µg/ml, respectively. Limits of quantitation for 4'-hydroxyflurbiprofen and flurbiprofen in urine and plasma were 0.5 µg/ml in urine and 0.05 µg/ml and 0.25 µg/ml, respectively, in plasma. A representative subject log plasma concentration versus time curve in Figure 6.6 shows the ability of this

assay to sensitively detect flurbiprofen and low 4'-hydroxyflurbiprofen concentrations in plasma. In addition, in 12 subjects receiving flurbiprofen orally, the average urinary recovery of total drug after acid cleavage of glucuronide metabolite was 21 mg out of a 50 mg dose, or about 42% (data not shown).

Calibration and linearity. Calibration curves were constructed using seven standard concentrations in urine or plasma. Curves were obtained daily for four days by plotting the peak-area ratios of flurbiprofen and 4'-hydroxyflurbiprofen to the IS against the corresponding nominal concentration. Linear calibration curves were generated by weighted (1/y) linear regression analysis. The mean $\pm$ s.d. coefficients of determination and slopes for 4'-hydroxyflurbiprofen and flurbiprofen in urine (n=4) were 0.996 ± 0.005 (slope = $6.84 \times 10^{-4} \pm 3.99 \times 10^{-5}$) and 0.994 ± 0.003 (slope = $1.92 \times 10^{-3} \pm 1.40 \times 10^{-4}$), respectively, while in plasma (n=4) they were 0.995 ± 0.004 (slope = $4.49 \times 10^{-3} \pm 1.78 \times 10^{-4}$) and 0.996 ± 0.001 (slope = $1.42 \times 10^{-2} \pm 2.45 \times 10^{-4}$), respectively. The lower limit of quantitation (LOQ) for flurbiprofen and 4'-hydroxyflurbiprofen in each matrix was selected as the lowest concentration of the standard curve

Precision and accuracy. The precision and accuracy of the urine and plasma assays were determined by three days of quality control analysis. Intra-day (n=12) and inter-day (n=3 days; n=24 samples) variability is shown in Tables 6.1 and Table 6.2, respectively. Analyte stability was demonstrated in both urine and plasma by subjecting the samples to three freeze-thaw cycles, and injecting the processed quality control samples every two hours over a 24 hour period. Three freeze-thaw cycles had no effect on the stability of the analytes in both urine and plasma, as the accuracy of all low and high QC concentrations (n=3) for flurbiprofen and 4'-hydroxyflurbiprofen was within

15% of expected values (Tables 6.1 and 6.2). In addition, the coefficients of variation were below 11% for all LC and HC amounts (data not shown). Likewise, processed stability after processing was not a factor, as the accuracy of all low and high QC concentrations (n=12) was within 10% of expected values, and all coefficients of variations were within 8.5% (Tables 6.1 and 6.2).

6.4. Discussion

This study describes a rapid and sensitive HPLC method for the detection and quantitation of flurbiprofen and 4'-hydroxyflurbiprofen in urine and plasma. The method uses simple protein precipitation with acetonitrile followed by direct sample injection resulting in short sample preparation times. Quality control analysis indicates that the assay is precise (C.V.<14%) and accurate (within 15%). The urinary recovery data and the representative plasma concentration-time curve suggest that this method is sufficient for attaining accurate clinical pharmacokinetic data following single dose administration of flurbiprofen. This assay has been used to validate use of the flurbiprofen eight hour urinary recovery ratio ($FLRR = \frac{4'OHF}{4'OHF + F}$) as an *in vivo* probe of cytochrome P450 2C9 activity in human subjects ({Frye, Tracy, et al. 2000 2493 /id}, manuscript in preparation). Applications of this methodology will allow determination of relative CYP2C9 metabolizing capacity in individuals, phenotypic identification of individuals possessing mutant CYP2C9 alleles (e.g., *2 and *3 [10;11]) which result in altered metabolizing capacity and determination of the effects of disease states on CYP2C9 activity.

Table 6.1. Detection of Flurbiprofen and 4'-Hydroxyflurbiprofen in Urine

Intra- and Inter-day precision and accuracy for flurbiprofen and 4'-hydroxyflurbiprofen in urine

Compound	Concentration Added (µg/ml)	Found	C.V. (%)	% Deviation
<i>Intra-assay reproducibility^a</i>		Mean+/- S.D.		
Flurbiprofen	1.0	0.90+/-0.09	9.5	-10.0
	12.0	10.29+/-0.40	3.9	-14.2
	30	28.23+/-2.58	9.1	-6.0
4'-hydroxyflurbiprofen	1.0	1.12+/-0.06	5.8	10.0
	12.0	11.46+/-0.47	4.1	-4.2
	30	29.96+/-2.77	9.3	-0.3
<i>Inter-assay reproducibility^b</i>		Mean +/- S.E.		
Flurbiprofen	1.0	0.88+/-0.02	2.7	-10.0
	12.0	10.39+/-0.34	3.6	-13.3
	30	29.34+/-1.10	3.8	-2.3
4'-hydroxyflurbiprofen	1.0	1.09+/-0.03	3.0	10.0
	12.0	11.30+/-0.39	3.5	-5.8
	30	30.54+/-1.00	3.4	1.7
<i>Freeze-thaw stability^c</i>		Mean +/- S.D.		
Flurbiprofen	1.0	0.85+/-0.07	8.2	-14.5
	30	29.64+/-1.56	5.3	-1.2
4'-hydroxyflurbiprofen	1.0	1.01+/-0.04	4.0	1.0
	30	30.65+/-1.39	4.5	2.2
<i>Processing stability^d</i>		Mean +/- S.D.		
Flurbiprofen	1.0	0.92+/-0.03	3.3	-7.7
	30	31.14+/-0.76	2.4	3.8
4'-hydroxyflurbiprofen	1.0	1.01+/-0.02	2.0	0.8
	30	31.99+/-0.63	2.0	6.6

^{a,d}Twelve samples per concentration

^bSix samples per day per concentration for three days

^cThree samples per concentration

Table 6.2. Detection of Flurbiprofen and 4'-Hydroxyflurbiprofen in Plasma

Intra- and Inter-day precision and accuracy for flurbiprofen and 4'-hydroxyflurbiprofen in plasma

Compound	Concentration		C.V. (%)	%Deviation
	Added	Found		
	(µg/ml)			
<i>Intra-assay reproducibility^a</i>		Mean+/- S.D.		
Flurbiprofen	0.5	0.48+/-0.03	6.3	-4.0
	6.0	5.49+/-0.23	4.2	-8.3
	15	13.93+/-0.69	4.9	-7.3
4'-hydroxyflurbiprofen	0.1	0.12+/-0.02	13.7	16.7
	0.5	0.46+/-0.03	6.9	-8.0
	1.5	1.39+/-0.07	4.9	-6.7
<i>Inter-assay reproducibility^b</i>		Mean +/- S.E.		
Flurbiprofen	0.5	0.46+/-0.02	4.4	-8.0
	6.0	5.41+/-0.18	3.3	-10.0
	15	13.97+/-0.04	0.3	-6.7
4'-hydroxyflurbiprofen	0.1	0.10+/-0.02	14.3	1.0
	0.5	0.45+/-0.01	2.7	-10.0
	1.5	1.41+/-0.02	1.2	-6.7
<i>Freeze-thaw stability^c</i>		Mean +/- S.D.		
Flurbiprofen	0.5	0.49+/-0.01	2.0	-2.9
	15	14.51+/-1.41	9.7	-3.2
4'-hydroxyflurbiprofen	0.1	0.09+/-0.01	11.1	-6.9
	1.5	1.45+/-0.16	11.0	-3.3
<i>Processing stability^d</i>		Mean +/- S.D.		
Flurbiprofen	0.5	0.52+/-0.02	3.8	4.2
	15	14.11+/-0.55	3.9	-5.9
4'-hydroxyflurbiprofen	0.1	0.09+/-0.01	11.1	-8.6
	1.5	1.45+/-0.04	2.8	-3.1

^{a,d}Twelve samples per concentration

^bSix samples per day per concentration for three days

^cThree samples per concentration

Figure 6.1. Flurbiprofen Metabolism

Structures for flurbiprofen and its primary metabolite, 4'-hydroxyflurbiprofen.

Figure 6.2. HPLC Chromatogram of Flurbiprofen and 4'-Hydroxyflurbiprofen in Urine

Representative HPLC chromatogram illustrating the detection of 4'-hydroxy flurbiprofen (5 $\mu\text{g/mL}$), internal standard, and flurbiprofen (5 $\mu\text{g/mL}$) in urine.

Figure 6.3. HPLC Chromatogram from Blank Urine Sample

Representative HPLC chromatogram from a blank urine sample.

Figure 6.4. HPLC Chromatogram of Flurbiprofen and 4'-Hydroxyflurbiprofen in Plasma

Representative HPLC chromatogram illustrating the detection of 4'-hydroxy flurbiprofen (0.05 µg/mL), internal standard, and flurbiprofen (0.25 µg/mL) in plasma.

Figure 6.5. HPLC Chromatogram from Blank Plasma Sample

Representative HPLC chromatogram from a blank plasma sample.

Figure 6.6. Flurbiprofen Log Plasma Concentration Time Profile

Representative log plasma concentration-time profile for flurbiprofen and 4'-hydroxyflurbiprofen from a healthy subject who received a 50 mg dose of racemic flurbiprofen.

CHAPTER VII

Minimal *In vivo* Activation of CYP2C9-Mediated Flurbiprofen Metabolism by Dapsone

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7.1. Introduction

The CYP450 enzymes are one of the most important groups of enzymes involved in the biotransformation of hydrophobic compounds such as drugs, carcinogens, and other xenobiotics. The majority of CYP450 reactions are oxidations (e.g., hydroxylation) that exhibit Michaelis-Menten kinetics and competitive inhibition between substrates. As a result, most pharmacokinetic properties can be predicted with standard kinetic analyses, which may be helpful in the prediction of drug interactions. However, it has been shown that some CYP450 reactions show unusual enzyme kinetics such as activation, autoactivation, and substrate inhibition [1;2]. Though reactions involving atypical kinetics are most commonly associated with CYP3A4, they have also been noted with CYP2C9 [2]. CYP2C9 is responsible for metabolizing several clinically relevant drugs such as S-warfarin, phenytoin, and many of the NSAIDs [3]. One NSAID in particular, flurbiprofen, is metabolized exclusively by CYP2C9 [4;5], and thus serves as a selective *in vitro* probe for CYP2C9 activity. Furthermore, this CYP2C9 mediated metabolism of flurbiprofen is non-stereoselective [4;5], implying that administration of commercially available *racemic*-flurbiprofen would be feasible for *in vivo* studies of CYP2C9 metabolism. To this end, we have recently shown that flurbiprofen may also be useful as an *in vivo* probe for CYP2C9 activity [6]

Dapsone is a sulfone drug that has been used in the treatment of leprosy, and more recently for the treatment of Kaposi's sarcoma and *pneumocystis carinii* in patients with AIDS [7]. Dapsone itself is metabolized by CYP2C9 [CYP3A4 and CYP2E1 may also provide a contribution] [8-11], and has been shown to participate in a complex *in vitro* interaction with flurbiprofen, whereby it can activate CYP2C9-mediated flurbiprofen

metabolism [2]. The addition of dapsone *in vitro* can increase CYP2C9 mediated flurbiprofen metabolism by 50-100% in human liver microsomes and purified CYP2C9 enzyme, respectively [2]. This observation of CYP2C9 activation *in vitro* at concentrations of both dapsone and flurbiprofen observed *in vivo*, suggests that CYP2C9 might display cooperativity *in vivo* and thus leads to the hypothesis that dapsone may activate flurbiprofen metabolism (via CYP2C9) *in vivo*. In this study, flurbiprofen was administered in the presence and absence of low and high concentrations of dapsone, to evaluate whether the metabolism of flurbiprofen could be activated *in vivo* by dapsone.

7.2. Methods

Chemicals and reagents. *S*-Flurbiprofen, 4'-hydroxyflurbiprofen, and 2-fluoro-4-biphenyl acetic acid (internal standard) were gifts from the Pharmacia, Inc. (Kalamazoo, MI, USA). Dapsone was obtained from Sigma Chemical Co. (St Louis, MO, USA) and monoacetyl-dapsone was synthesized by the method of Uetrecht et al. [12]. All other chemicals and solvents were obtained from commercial sources and were of the highest purity available.

Inclusion/exclusion criteria. Twelve healthy subjects between 18 and 45 years of age participated in the study. The study protocol was approved by the Institutional Review Boards of both the University of Pittsburgh and West Virginia University and all subjects gave written, informed consent. The subjects consisted of non-smoking men and women who underwent screening tests based on history, physical examination and routine biochemical and urinalysis tests. Women must not have been pregnant at the time of the study, and all subjects must have abstained from alcohol (of any form) for 8 days

and any caffeine containing products for 48 hours before and during the period of study. Subjects were specifically questioned about the last time they consumed alcohol or any caffeine containing products.

Flurbiprofen-dapsone pharmacokinetic study. The pharmacokinetics of flurbiprofen were evaluated before (visit A) and after the first and seventh dose(s) of dapsone 100mg (visits B and C, respectively). Visits A and B (before and after dapsone) were separated by at least 2 weeks. *Racemic*-Flurbiprofen (Ansaid[®]) 50mg was administered orally after an overnight fast on each occasion and given 2 hours after the single or seventh dose of dapsone (Avlosulfon[®]). Prior to drug administration, 40 ml of blood was obtained. Following drug administration, blood samples (20 ml with sodium EDTA) were obtained at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 hours. Total voided urine was collected before and at the following time intervals after flurbiprofen administration: 0-2 hr, 2-4 hr, 4-6 hr, 6-8 hr, 8-10 hr, 10-12 hr, and 12-24 hr. Subjects were kept at the Clinical Research Unit for 12 hours during each study and returned the next morning for the 24 hr blood sample draw and to provide the overnight (12-24 hr) urine collection. Plasma was obtained from the blood samples by centrifugation and together with aliquots of the urine samples, stored frozen at -20°C until analysis.

Urine 4'-hydroxyflurbiprofen and flurbiprofen concentration analysis. Plasma and urinary concentrations of 4'-hydroxyflurbiprofen and flurbiprofen were measured using the method of Hutzler et al., [13]. Samples were prepared using 50µl of urine, followed by addition of 400 µl of purified water and 100 µl 6M HCl. The samples were then incubated at 90°C for 30 minutes to facilitate deconjugation of the glucuronides of flurbiprofen and its 4'-hydroxy metabolite. Once cooled, 500µl of acetonitrile containing

internal standard (500ng of 2-fluoro-4-biphenyl acetic acid) was added. Samples were then vortexed and centrifuged at 10,000 rpm for 5 minutes. Next, 150 µl of the supernatant was transferred to autosampler vials and injected onto the HPLC. The HPLC system consisted of a Waters® 501 HPLC pump, a Waters® 717 autosampler and a Waters® 470 fluorescence detector set at an excitation wavelength of 260nm and an emission wavelength of 320nm. The mobile phase consisted of acetonitrile:20mM K₂HPO₄, pH 3.0 (40:60) pumped at 1mL/min through a Brownlee Spheri-5® C₁₈ 4.6 x 100mm column. The inter-day coefficients of variation for 4'-hydroxyflurbiprofen and flurbiprofen in urine were less than 5%.

Plasma 4'-hydroxyflurbiprofen and flurbiprofen concentration analysis. Plasma samples were prepared by adding 200 µl internal standard solution (180 ng/ml 2-fluoro-4-biphenylacetic acid in acetonitrile) to 100 µl plasma, followed by brief vortexing. After 200 µl acetonitrile was added, samples were vortexed and centrifuged for 10 minutes. Next, 100µl of sample was transferred to HPLC autosampler vials, and 50µl was injected. The mobile phase was pumped in a gradient fashion through a Thomson 20-20 column C₁₈ 4.6 x 200mm 5µm column. Mobile phase A consisted of acetonitrile, containing 0.1% acetic acid, and mobile phase B consisted of 0.1% acetic acid. Using a Hewlett Packard 1050 HPLC system pumping at 1mL/min, the gradient was initiated at a ratio of 30%A:70%B and altered in a linear fashion to 100% A over 20 minutes. The system was returned to initial conditions over 6 minutes and then allowed to equilibrate for 5 minutes, for a total run time of 31 minutes. Samples were quantitated with fluorescence detection using an excitation wavelength of 260nm and an emission

wavelength of 320nm. The inter-day coefficients of variation for 4'-hydroxyflurbiprofen and flurbiprofen in plasma were 14.3% or less.

Plasma dapsone and N-acetyldapsone concentration analysis. The concentrations of dapsone and N-acetyl dapsone were determined by HPLC as described previously {May, Arns, et al. 1992 1140 /id}. The inter-day coefficients of variation for dapsone and N-acetyldapsone in plasma were 13% or less.

Data analysis. Plasma concentration-time data were analyzed by non-compartmental methods. Pharmacokinetic parameters including half-life, clearance, area under the concentration-time curve (AUC) and metabolite formation clearance were estimated using WinNonlin (Pharsight, Palo Alto, CA). The AUC for flurbiprofen and 4'-hydroxyflurbiprofen were determined using the trapezoidal rule with extrapolation to infinity. Flurbiprofen apparent oral clearance was calculated as $Cl/F = \text{Dose}/\text{AUC}$. 4'-hydroxyflurbiprofen formation clearance (4'-OHF FCL) was calculated as:

$$CL_{FM}^{4OH} = \frac{\text{Urinary excretion of 4'-OHF (24hours)}}{\text{AUC Flurbiprofen}_{(0-\infty)}}$$

The AUC (0-12 hours) for dapsone and N-acetyl dapsone was also determined using the trapezoidal rule. Mean concentrations were calculated by dividing the AUC of these compounds by the time interval (12 hours).

Statistical analysis. Data are presented as mean $\pm$ s.d. Data were analyzed by repeated measures analysis of variance followed by Tukey's multiple comparison test. Clearance values were log transformed prior to analysis. Differences were regarded as statistically significant when P values were ≤ 0.05 . All analyses were performed using SAS software (v. 6.12).

7.3. Results

The apparent oral clearances of flurbiprofen during each of the treatment periods are presented in Figure 7.1. The apparent oral clearance of flurbiprofen was unchanged when given concomitantly with a single dose of dapsone but increased slightly (~11%) ($P<0.02$) after seven days of dapsone dosing (Table 7.1). It is of note that though there were substantial differences in baseline flurbiprofen apparent oral clearance values among the patients, the reproducibility of changes within subjects were similar in extent (Figure 7.1). Thus, averaging of the data somewhat obscures the changes noted within an individual patient. The remaining pharmacokinetic parameter estimates for all three visits are listed in Table 7.1.

To assess whether dapsone affected the glucuronidation of flurbiprofen and its 4'-hydroxyflurbiprofen metabolite, urine samples from Visits A and C (without and with dapsone) from three patients were analyzed with and without acid hydrolysis of the glucuronides (data not shown). The ratio of 4'-hydroxyflurbiprofen recovered to total parent and metabolite recovered from Visit A (no dapsone) did not differ regardless of whether unconjugated flurbiprofen and metabolite or total flurbiprofen and metabolite after hydrolysis were measured. Furthermore, the same equality was found in the hydrolyzed and un-hydrolyzed urine samples from patients taking dapsone concurrently. Likewise, measuring unconjugated drug did not alter the calculations of percent activation as compared to measuring total drug excreted. These findings suggest that dapsone had no effect on excretion of the flurbiprofen and metabolite glucuronides.

The AUC (0-12h) of dapsone and N-acetyldapsone estimated by the trapezoidal rule is shown in Table 7.2. Average plasma dapsone levels after single and multiple

doses were $5 \pm 2\mu\text{M}$ and $11 \pm 4\mu\text{M}$, respectively (Table 7.2). Likewise, mean N-acetyldapsone levels in plasma were $2 \pm 1\mu\text{M}$ after a single dose and $4 \pm 2\mu\text{M}$ after seven daily doses (Table 7.2). These average concentrations of dapsone ($5\mu\text{M}$ and $11\mu\text{M}$) were well within the range of dapsone concentrations shown to produce activation of CYP2C9 mediated flurbiprofen metabolism *in vitro* [2]. Furthermore, there was no correlation between N-acetylator status (slow or rapid acetylators) and degree of activation as measured by the flurbiprofen recovery ratio (data not shown).

7.4. Discussion

The present study evaluated the ability of dapsone to activate (interact cooperatively with) flurbiprofen metabolism to 4-hydroxyflurbiprofen *in vivo*. Flurbiprofen is an NSAID that is exclusively metabolized by CYP2C9 via 4'-hydroxylation [4;5]. As a result, it may be used as a selective probe to monitor the activity of CYP2C9 under various *in vitro* conditions. More recently, it has been determined that flurbiprofen may also be used as a phenotypic measure of CYP2C9 activity *in vivo* [6]. Results of the present study demonstrate minimal activation of flurbiprofen metabolism *in vivo* by dapsone, as reflected by an increase in flurbiprofen apparent oral clearance.

In vitro experiments utilizing microsomes and purified CYP2C9 enzyme have shown that dapsone activates 2C9-mediated flurbiprofen 4'-hydroxylation [2]. This activation phenomenon was first discovered in microsomes, where a 50% activation was observed, with maximum activation occurring at approximately $10\mu\text{M}$ dapsone concentrations [2]. Studies using a purified CYP2C9 reconstituted enzyme system were

also conducted, with flurbiprofen metabolism being doubled in the presence of low concentrations of dapsone, and the maximum effect again at 10 μ M dapsone [2]. Our laboratory has determined that this activation is not an isolated case, as observations that dapsone can activate CYP2C9 mediated metabolism of the NSAID naproxen *in vitro* have also been made [2].

Activation of CYP-mediated metabolism has primarily been associated with CYP3A4. Studies by Schwab et al. [14] and Shou et al. [1] have shown that purified CYP3A enzymes are activated by 7,8-benzoflavone. Likewise, purified CYP3A4-mediated meloxicam metabolism was activated by quinidine in a dose-dependent manner, 160% at 10 μ M and 510% at 100 μ M compared to control experiments [15]. Most recently, Tang et al. demonstrated in rhesus monkeys substantial (2-fold) *in vivo* activation of CYP3A4 metabolism of diclofenac by quinidine [16]. More recent evidence from Korzekwa et al., [2] and Ekins et al. [17] suggest activation is not unique to CYP3A4 and that other isoforms may undergo activation as well.

Based on *in vitro* data showing activation of CYP2C9, we hypothesized that similar findings might be observed *in vivo* using the CYP2C9 probe flurbiprofen. Though the direction of the observed change in flurbiprofen apparent oral clearance is consistent with this hypothesis, the *in vivo* interaction is of much lesser magnitude than observed *in vitro*. At this time it is unclear why the degree of activation observed *in vitro* is of a greater magnitude than observed *in vivo*. One factor that may contribute to the decreased activation *in vivo* is protein binding. Recent work by McLure et al. [18] has demonstrated that the binding of naproxen (an NSAID structurally related to flurbiprofen) exhibits very little non-specific binding to microsomal (enzyme) protein *in*

vitro. The *in vitro* binding of dapsone in microsomal preparations is unknown. However, both flurbiprofen (99.95%) [19;20], and dapsone (~70-90%) [21] are substantially bound to serum albumin *in vivo*. If a drug is highly protein bound, then the amount of free drug available for metabolism and/or activation may be small. Since total levels of dapsone were measured, and dapsone is 70-90% protein bound, then we may assume that only about 20% of the dapsone was available to act on CYP2C9 *in vivo*, potentially resulting in less activation compared to *in vitro*. As previously mentioned, the average concentrations of dapsone in the subjects after one and seven doses were 5 ± 2 μM and 11 ± 4 μM , respectively, which corresponds to free dapsone concentrations of ~1 μM and 2 μM , respectively, assuming 80% protein binding. By taking the average $\text{AUC}_{0-\infty}$ of flurbiprofen from visit A, the average flurbiprofen concentration can be calculated as being approximately 7 μM , resulting in an unbound concentration of 0.0035 μM . These estimated unbound concentrations result in an average *in vivo* unbound dapsone/flurbiprofen molar ratio of ~600 after seven days of dapsone therapy. Even without knowing the *in vitro* non-specific binding of dapsone, it is obvious that the unbound dapsone/flurbiprofen ratio *in vitro* is less than 600 suggesting that though protein binding may play some role in the lack of *in vitro-in vivo* correlation, it is probably not the entire explanation.

Dapsone and flurbiprofen both have several routes of metabolism, which may also have affected our *in vivo* observations. Dapsone is metabolized via both oxidative and conjugative pathways, forming the hydroxylamine of dapsone and the N-acetylated metabolites monoacetyldapsone and hydroxymonoacetyldapsone [22;23]. *In vivo*, dapsone is concurrently undergoing both oxidative and conjugative metabolism, resulting

in less dapsone available to act upon the CYP2C9 isoform and potentially reducing activation, as compared to our *in vitro* experiments [2] in which only phase I pathways were operational. Besides dapsone metabolism, another potential confounder in these *in vitro-in vivo* correlations is that flurbiprofen also undergoes glucuronidation [24;25], which may serve as a competitive reaction, lowering the effective flurbiprofen concentration available for oxidation, even though dapsone does not appear to affect flurbiprofen glucuronidation. Thus, protein binding and additional metabolism pathways likely play a role in the disparity between *in vitro* and *in vivo* degrees of flurbiprofen activation by dapsone. However, it is possible that other unidentified factors may also participate in the modulation of activation *in vivo*.

In conclusion, the activation of flurbiprofen metabolism by dapsone observed *in vitro* has been observed in man. However, the activation does not appear to occur at the same magnitude. Thus, it can be expected that co-administration of dapsone with other CYP2C9 substrates would not result in clinically significant interactions. It remains possible that *in vivo* study of compounds exhibiting greater *in vitro* activation than flurbiprofen or involving other CYP isoforms (e.g., CYP3A4 with its broad substrate specificity) may result in a greater change in clearance. An improved understanding of this activation phenomenon should permit a better assessment of its potential clinical significance.

Table 7.1. Flurbiprofen Pharmacokinetic Parameter Estimates

Mean flurbiprofen kinetic parameter estimates.

Variable	Units	Visit A	Visit B	Visit C
AUC _{0-∞}	µg/L*hr	43.8±13.8	39.3±9.4	38.3±8.5
Cl/F	L/hr	1.23±0.32	1.33±0.29	1.37±0.30*
C _{max}	µg/ml	10.5±2.9	9.9±4.2	10.0±3.4
T _{1/2}	hours	3.7±1.0	3.6±1.3	3.3±0.6
T _{max}	hours	1.5±0.9	1.8±1.0	1.4±0.8
V _d /F	L	6.2±1.2	6.9±3.2	6.5±2.2
4'-OH FCl	L/hr	0.38±0.14	0.39±0.13	0.40±0.16

Data are mean values ±SD

Visit A = Flurbiprofen 50mg alone.

Visit B = Flurbiprofen 50mg + Dapsone 100mg.

Visit C = Flurbiprofen 50mg after 7 days of Dapsone 100mg daily.

AUC_{0-∞}, area under the plasma concentration-time curve extrapolated to infinity; Cl/F, clearance; T_{1/2}, half-life; 4'-OH FCl, 4'-hydroxy flurbiprofen formation clearance.

*P<0.02

Table 7.2. Plasma Levels of Dapsone and N-acetyl Dapsone

Mean plasma levels for dapsone and N-acetyl dapsone.

Visit	Dapsone		N-acetyl dapsone	
	B	C	B	C
AUC (0-12h)µM*hr	59.5±18.8	130.5±45.9	24.0±15.0	47.8±24.5
C _{max} (µM)	7.5±2.2	14.5±4.9	3.3±2.3	5.9±3.4
C _{average} (µM)	5.0±1.6	10.9±3.8	2.0±1.2	4.0±2.0

Dapsone dose =100mg

B=single dose

C=7th dose

Figure 7.1. Flurbiprofen Oral Clearance in 12 Healthy Subjects

Comparison of flurbiprofen oral clearance in the 12 subjects when receiving either flurbiprofen alone (Visit A), flurbiprofen and single dose dapsone (Visit B) or seven days of dapsone therapy and then flurbiprofen (Visit C).

Summary and Conclusions

Over the past several years, it has come to our attention that CYP450 enzymes do not always behave as would be expected. Many display kinetics that are not consistent with the Michaelis-Menten model, resulting in data that is often hard to interpret and correlate to the *in vivo* situation. As a result, it is important that we understand, as best we can, the underlying causes of atypical kinetics. This understanding will help to better predict when atypical kinetics are likely to occur. Historically, CYP3A4 has been the focus of study because it has presented the most examples of atypical kinetics. However, it is now being discovered that other isoforms often show atypical kinetic profiles as well. Our lab has discovered that dapsone can activate CYP2C9-mediated metabolism of several compounds. This was first discovered in human liver microsomes, but little was known about the underlying causes. The work done since that initial discovery, and reported here, has shed some light on this subject and provided valuable information regarding the mechanism of activation of CYP2C9.

It was first discovered that dapsone activated flurbiprofen 4'-hydroxylation, but we know now that naproxen demethylation and piroxicam 5'-hydroxylation are also activated by dapsone, indicating that multiple CYP2C9 substrates can be activated by dapsone. Kinetic studies exploring the activation of these substrates indicates that activation of CYP2C9 may be explained by a two-site binding model, which suggests that both dapsone and substrate may fit in the active site simultaneously, both having access to the active oxygen. In addition, we have the capability of fitting activation kinetic data to a two-site effector model equation, which gives α and β values indicative of activation.

Next we have shown that the effector structural requirements are rather strict, which is consistent with other specific protein-substrate interactions. It is evident from

these dapstone analog studies that the best activators possess a sulfone moiety, preferably directly between two benzene rings. Para positions on these benzene rings also are important, as replacement of an amino group (dapstone) with a nitro group (4-NPSA) results in inhibition of CYP2C9. This small structural change in 4-NPSA, linked to the substantial change in enzyme activity in its presence, suggests that the effector site that causes activation is very specific, and may be in the active site. To study the effects of active site changes on activation of dapstone, we have studied the *2 and *3 allelic variants and F114L mutant of CYP2C9. Results suggested that the F114 residue may play a role in activation, as 5-fold activation of flurbiprofen 4'-hydroxylation was observed in this mutant, compared to 1.5-fold in wild-type CYP2C9. In addition, piroxicam 5'-hydroxylase activity was higher in the F114L mutant than wild-type CYP2C9 in the absence of dapstone, indicating that active site conformation alone contributes to enzyme activity.

In addition, it has been found that CYP2C9 may be activated by more than one mechanism. Hydroxy-dapstone has been found to activate flurbiprofen 4'-hydroxylation, although differently from dapstone. Hydroxy-dapstone activates CYP2C9 solely through a $V_{\max}$ effect, with no effect on K_m , whereas dapstone affects both $V_{\max}$ and K_m . These effects were confirmed by spectral binding studies which showed the direct effect of dapstone on the binding affinity for flurbiprofen, while hydroxy-dapstone had no effect on affinity for flurbiprofen. This $V_{\max}$ effect by hydroxy-dapstone may be the result of disrupting ordered water molecules within the active site, resulting in decreased uncoupling of the P450 cycle and enhanced product formation. This suggests that multiple factors are likely involved in activation of CYP2C9 activity. We have also

found from CYP2C9/19 chimeric and CYP2C19 mutant enzymes the important regions/residues for catalytic activity towards flurbiprofen, naproxen, and piroxicam. Additionally, only the CYP2C9/19 (340-490) chimera was able to be activated to any extent by dapsone. Results from the same study suggest that dapsone may bind in SRS 1 of CYP2C9, but this has yet to be confirmed. Lastly, the orientation of ibuprofen is apparently changed in the presence of dapsone, as a different ratio of 3-hydroxy to 2-hydroxy ibuprofen is produced in the presence of dapsone, indicating the active site conformation effects of dapsone on CYP2C9.

One of the ongoing arguments regarding effector binding and atypical kinetics is exactly where the effector site is. Most data presented in the literature suggests that the effector site is in the active site, while others still believe in the presence of an allosteric site, which upon binding by effector causes a conformational change in the protein. None of our studies have conclusively shown the presence of the effector site in the active site. However, we have shown some pretty compelling kinetic and structural evidence to support this theory, including the ability to measure dapsone metabolite formation in the presence of flubiprofen, indicating dapsone's presence in the active site (chapter 2). Despite the information collected thus far to help in the understanding of activation of CYP2C9, there is still much to be studied before we can truly state that we fully understand atypical kinetic profiles. It is also possible that overall protein conformational changes are contributing to the observed activation of CYP2C9, which is an area of focus for the future. The information reported here, while important, is just the beginning.

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5. Tracy TS, Marra C, Wrighton SA, Gonzalez FJ, Korzekwa KR. Studies of flurbiprofen 4'-hydroxylation: Additional evidence suggesting the sole involvement of cytochrome P450 2C9. *Biochem Pharmacol* 1996; **52**:1305-1309.
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1. Shou M, Grogan J, Mancewicz JA *et al.* Activation of CYP3A4: Evidence for the simultaneous binding of two substrates in a cytochrome P450 active site. *Biochemistry* 1994; **33**:6450-6455.
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12. Uetrecht JP, Zahid N, Shear NH, Biggar WD. Metabolism of dapsone to a hydroxylamine by human neutrophils and mononuclear cells. *J Pharmacol Exp Ther* 1988; **245**:274-279.
13. Hutzler JM, Frye RF, Tracy TS. Sensitive and specific high-performance liquid chromatographic assay for 4'-hydroxyflurbiprofen and flurbiprofen in human urine and plasma. *J Chromatogr B Biomed Sci Appl* 2000; **749**:119-125.
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18. McLure JA, Miners JO, Birkett DJ. Nonspecific binding of drugs to human liver microsomes. *Br J Clin Pharmacol* 2000; **49**:453-461.
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22. Israili ZH, Cucinell SA, Vaught J, Davis E, Lesser JM, Dayton PG. Studies of the Metabolism of Dapsone in Man and Experimental Animals: Formation of N-Hydroxy Metabolites. *J Pharmacol Exp Ther* 1973; **187**:138-151.
23. Gelber R, Peters JH, Gordon GR, Glazko AJ, Levy L. The polymorphic acetylation of dapsone in man. *Clin Pharmacol Ther* 1971; **13**:225-238.
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Curriculum Vitae

JAMES M. HUTZLER

Birth date: March 10, 1974

Place of Birth: Winchester, Virginia

GENERAL INFORMATION

Home Address: 159 Mountaineer Village
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ACADEMIC TRAINING

Doctor of Philosophy (Ph.D.):

Drug Metabolism/Pharmacokinetics, School of Pharmacy,
West Virginia University, Morgantown, WV, December 2001

Dissertation Title: Factors Affecting CYP2C9-Mediated Metabolism

Dissertation Advisor: Dr. Timothy S. Tracy, Associate Professor, School of
Pharmacy, West Virginia University

Graduate Level Coursework: Biochemistry, Protein Biochemistry, Statistics,
Pharmacokinetics, Advanced Pharmacokinetics, Pharmaceutical Sciences (Bench
to Shelf), Chemical Properties of Drugs, Instrumental Analysis, Pharmacology I
and II, Drug Metabolism

Bachelor of Science: Shepherd College, Shepherdstown, West Virginia, 1996

Major: Biology

Minor: Chemistry

ACADEMIC AWARDS

1999-2001	AFPE (American Foundation for Pharmaceutical Education) Pre-Doctoral Fellowship
1998	Rho Chi Honorary Society
1997-1998	Mylan Pharmaceuticals Fellow
1996	Outstanding Senior in Biology (Shepherd College)
1996	McMurrin Scholar (Shepherd College)

RESEARCH SKILLS

In vitro incubation studies with cytochrome P450 enzymes (microsomes, expressed, and purified enzyme systems)
HPLC analysis with UV, Fluorescence, and PDA detection
Quantitation with ESI and APCI LC-MS (Micromass ZMD)
Spectrophotometric methods (obtaining CYP450 binding spectra)
Processing urine and plasma clinical samples for HPLC analysis
Noncompartmental pharmacokinetic analysis using WinNonlin
Enzyme kinetic modeling using programs such as Axum 6.0, Sigma Plot 2001 with Enzyme Kinetics Module, and Prism (Graphpad)
Continuous fluorometric monitoring
Mechanism-based CYP450 inactivation

COMPUTER SKILLS

Microsoft Word, Excel, Powerpoint, WINNONLIN, Axum 5.0 and 6.0, Sigma Plot, Prism

TEACHING EXPERIENCE

Problem Based Learning (PBL)-served as PBL group facilitator to first and second year pharmacy students. PBL cases were used to teach students how to apply their basic pharmaceutical science knowledge to solve clinical problems.

PUBLICATIONS

Hutzler JM, Frye RF, Tracy TS. Sensitive and Specific High-Performance Liquid Chromatographic Assay for 4'-Hydroxy-flurbiprofen and Flurbiprofen in Human Urine and Plasma. *Journal of Chromatography B* 2000;749:119-125.

Scharman EJ, Hutzler JM, Rosencrance JG, Tracy, TS. Single Dose Pharmacokinetics of Syrup of Ipecac. *Therapeutic Drug Monitoring* 2000;22:566-573.

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Tracy TS, Hutzler JM, Haining RL, Rettie AE, Hummel MA, Dickmann LJ. Polymorphic Variants (CYP2C9*3 and CYP2C9*5) and the F114L Active Site Mutation

of CYP2C9: Effect on Atypical Kinetic Metabolism Profiles. *Drug Metabolism and Disposition*. Accepted for publication.

Hutzler JM, and Tracy TS. Atypical Kinetic Profiles in Drug Metabolism Reactions: Minireview. *Drug Metabolism and Disposition*. Accepted for publication.

ABSTRACTS

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Frye, R.F., Tracy, T.S., Hutzler, J.M., Korzekwa, K.R., Cannon, Y., Pauli, M., Huang, S-M., Branch, R.A. Flurbiprofen as a Selective In Vivo Probe of CYP2C9 Activity. American Society for Clinical Pharmacology and Therapeutics Annual Meeting: Los Angeles, CA, March 2000 (Clinical Pharmacology and Therapeutics 2000:67;109)

Tracy, T.S., Rettie, A.E., Haining, R.L., Goldstein, J., Metz, C.A., Hutzler, J.M., Korzekwa, K.R. Metabolism of Flurbiprofen, Naproxen and Piroxicam by Cytochrome P450 2C Isoforms: CYP2C9 Selectivity, Atypical Kinetics and Effect of Allelic Variants on the Kinetic Profile. Proceedings of the 9th North American International Society for the Study of Xenobiotics Meeting, Volume 15, page 131.

Ramachandran, V., Kostrubsky, V.E., Dorko, K., Esplen, J., Hutzler, J.M., Strom, S.C., Tracy, T.S., Venkataramanan, R. Assessment of the Induction of CYP2C9 by Rifampicin and Dexamethasone in Primary Cultures of Human Hepatocytes. American Association of Pharmaceutical Scientists Annual Meeting, Nov. 1999.

Hutzler, J.M., Rettie, A.E., Haining, R.L., Tracy, T.S. Flurbiprofen Hydroxylation by Chimeric P450 2C9/19 Enzymes. American Society for Clinical Pharmacology and Therapeutics Annual Meeting: San Antonio, TX, March 1999 (Clinical Pharmacology and Therapeutics 1999:65;164).

Hutzler, J.M. and Tracy, T.S. Activation of CYP2C9 by Dapsone and Analogs. Proceedings of the 10th North American International Society for the Study of Xenobiotics Meeting: Indianapolis, IN, October 2000.

*Selected as finalist for best graduate student poster.

Hutzler, J.M., Rettie, A.E., Haining, R.L., Tracy, T.S. Dapsone Activation of CYP2C9 Allelic Variants. Proceedings of the 10th North American International Society for the Study of Xenobiotics Meeting: Indianapolis, IN, October 2000.

Tracy, T.S., Hutzler, J.M., Hamman, M.A., Hall, S.D. Dapsone Activation of CYP2C9 Mediated Ibuprofen Metabolism: Regio- and Stereoselective Effects. Proceedings of the

10th North American International Society for the Study of Xenobiotics Meeting:
Indianapolis, IN, October 2000.

Hutzler, J.M., Hauer, M.J., Tracy, T.S. Dapsone Activation of Multiple CYP2C9
Substrates: Evidence for a Two-Site Model. 31st Gordon Research Conference (Drug
Metabolism): Holderness School, Plymouth, NH, July 2001.

PROFESSIONAL SEMINARS

Does Dapsone-Mediated Activation of Flurbiprofen Metabolism by CYP2C9 Occur In
Vivo? Center for Clinical Pharmacology, University of Pittsburgh, Pittsburgh, PA.
February 11, 2000.

Exploring the Mechanism of CYP2C9 Activation by Dapsone. Center for Clinical
Pharmacology, University of Pittsburgh, Pittsburgh, PA. April 5, 2001.

Dapsone Activation of Multiple CYP2C9 Substrates: Evidence For a Two-Site Binding
Model. 31st Gordon Research Conference (Drug Metabolism), Holderness School,
Plymouth, New Hampshire. July 11, 2001.

PROFESSIONAL DEVELOPMENT WORKSHOPS ATTENDED

Waters Corp. Reversed Phase HPLC Column Selection & Optimization. University of
Pittsburgh, June 15, 1999.

PROFESSIONAL INTERNSHIPS

Pharmacia Corp., Kalamazoo, Michigan (June-August, 2000)

Supervisor: Richard A. Voorman, Ph.D.

Project: Continuous Fluorometric Monitoring of Mechanism-Based Inactivation of
CYP1A2 by Furafylline.