Published in final edited form as:

Arch Biochem Biophys. 2008 July 15; 475(2): 175–183.

Substrate Proton to Heme Distances in CYP2C9 Allelic Variants and Alterations by the Heterotropic Activator, Dapsone

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Abstract

CYP2C9 polymorphisms result in reduced enzyme catalytic activity and greater activation by effector molecules as compared to wild type protein, with the mechanism(s) for these changes in activity not fully elucidated. Through T₁ NMR and spectral binding analyses, mechanism(s) for these differences in behavior of the variant proteins (CYP2C9.2, CYP2C9.3 and CYP2C9.5) as compared to CYP2C9.1 were assessed. Neither altered binding affinity nor substrate (flurbiprofen) proton to heme-iron distances differed substantially among the four enzymes. Co-incubation with dapsone resulted in reduced substrate proton to heme-iron distances for all enzymes, providing at least a partial mechanism for the activation of CYP2C9 variants by dapsone. In summary, neither altered binding affinity nor substrate orientation appear to be major factors in the reduced catalytic activity noted in the CYP2C9 variants, but dapsone co-incubation caused similar changes in substrate proton to heme-iron distances suggesting at least partial common mechanisms in the activation of the CYP2C9 forms.

Keywords

cytochrome P450; CYP2C9; kinetics; activation; proton distance; T₁ NMR; genetic polymorphism; variant; binding affinity

Introduction

Allelic variant forms of polymorphic drug metabolizing enzymes, especially CYP2C9, often display altered kinetics from the wild type enzyme. Most commonly, these variants exhibit a reduction in the rate of metabolism. Many *in vivo* and *in vitro* examples of these alterations in enzyme activity exist. For example, Dickmann et al. demonstrated that CYP2C9.3 and CYP2C9.5 both exhibit reduced metabolizing activity toward (S)-warfarin, diclofenac, and

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lauric acid metabolism [1]. *In vivo*, patients expressing a *CYP2C9* allelic variant form (either *2 or *3) require lower than normal doses of warfarin and phenytoin to prevent toxicity due to reduced metabolism [2;3]. Changes in kinetic profile can also occur with *CYP2C9* allelic variants as has been observed with naproxen demethylation exhibiting a linear kinetic profile with *CYP2C9.3* and *CYP2C9.5* as compared to the biphasic profile observed with *CYP2C9.1* [4]. Also, piroxicam exhibited a substrate inhibition profile when metabolized by *CYP2C9.1* and *CYP2C9.3*, but a hyperbolic kinetic profile was evident with *CYP2C9.5* [4]. These allelic variants result from single nucleotide polymorphisms encoding for single amino acid changes in the protein. These amino acid changes may affect substrate turnover either directly or indirectly through effects on substrate binding, alterations in electron transport or active site waters affecting P450 cycle function or changes in active site conformation [5].

Of the *CYP2C9* variant forms discovered to date, three of the most extensively characterized are *CYP2C9.2*, *CYP2C9.3*, and *CYP2C9.5*. The R144C substitution in *CYP2C9.2* is outside of the active site and was originally proposed to affect P450 reductase binding [6], though more recent studies do not support this hypothesis and instead suggest that it affects catalytic cycle functioning through alterations in shunting products and potentially water matrix disruption [5]. The I359L and D360E changes in *CYP2C9.3* and *CYP2C9.5*, respectively, are conservative amino acid substitutions at positions thought to not be in direct contact with substrates in the active site cavity [7;8]. However, the dramatic reduction (approximately 4% for *CYP2C9.3* and 10% for *CYP2C9.5* of wild type activity) [4;9] in turnover caused by these two changes suggests that alterations in interactions with other amino acids may occur that could modify the active site topology and subsequently substrate binding.

Since these variant forms of *CYP2C9* alter the rate of substrate metabolism, it is possible that these amino acid changes may also impact the effects of inhibitor/activator (effector) molecules. Drug-drug interactions involving *CYP2C9* have been shown to be substrate, effector, and enzyme variant form dependent, *in vitro*. In an example of variant-dependent effects, dapsone activation of metabolism of the nonsteroidal anti-inflammatory drugs (NSAIDs), naproxen and flurbiprofen, occurred to a much greater extent in *CYP2C9* allelic variants than in the wild type enzyme [9]. With respect to substrate-dependent effects, in a study of dapsone analogs and their effects on *CYP2C9*-mediated metabolism, Hutzler et al. reported that phenylsulfone was a good activator of flurbiprofen metabolism but not of naproxen metabolism [10]. More recently, substrate-dependent inhibition of both *CYP2C9.1* and *CYP2C9.3* was reported [11]. Thus, one cannot simply generalize that all effectors will produce the same interaction with all substrates or that the degree of effect will be similar.

To examine the effects of *CYP2C9* variant proteins (*CYP2C9.1*, *CYP2C9.2*, *CYP2C9.3* and *CYP2C9.5*) on substrate orientation and proton-heme iron distances, NMR derived T_1 relaxation studies were previously conducted with the probe substrate flurbiprofen. In addition, co-incubation of flurbiprofen with dapsone not only results in heteroactivation but also causes the flurbiprofen protons to move closer to the heme iron in wild type protein [12]. Thus, we also examined the effects of dapsone on flurbiprofen distance and orientation in these same variants. In addition, binding affinity was assessed through UV/Vis spectral binding studies. The purpose of these studies was to gain additional information on mechanisms involved in altered catalytic activity with the *CYP2C9* variants and differences in degree of heteroactivation noted among the variant proteins.

Materials and Methods

Chemicals

D₂O, polyvinylpyrrolidone, sodium dithionite, *racemic*-flurbiprofen, dapsone, and dilauroylphosphatidylcholine were purchased from Sigma-Aldrich (St. Louis, MO). Centricon

MW cutoff filters were purchased from Millipore (Billerica, MA). Potassium phosphate, and EDTA were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from commercial sources and were of the highest purity available.

Enzyme Expression and Purification

CYP2C9.1 and CYP2C9.3 were expressed in *E. Coli* according to established methods [13]. Purified CYP2C9.2 and CYP2C9.5 proteins were provided by Dr. Allan Rettie from the University of Washington.

Spectral binding

Spectral binding studies to measure enzyme-substrate affinity were performed as previously reported [14]. Briefly, 300 pmol of enzyme along with 0.2 µg/pmol dilauroylphosphatidyl choline (200 nm vesicles) was placed into the sample and reference cuvette. Aliquots (5 µL) of flurbiprofen dissolved in 50 mM pH 7.4 potassium phosphate buffer were added to the sample cuvette while 5 µL of 50 mM pH 7.4 potassium phosphate buffer was added to the reference cuvette. After mixing, the sample and reference cuvettes were allowed to equilibrate for 3 minutes prior to spectral analysis. Spectra were recorded on an Aminco DW-2000 UV/Vis spectrophotometer with Olis modifications (Olis, Inc., Bogart, GA). The spectrophotometer was set to record spectra between 350 and 500 nm wavelengths with a slit width of 6.0 nm and scan rate of 100 nm/min. The temperature was held at a constant 28° C. The difference in absorbance between the peak (~390) and trough (~420) of the observed Type-I binding spectrum was calculated and plotted against flurbiprofen concentration. A binding constant (K_S) was determined by fitting the resulting data to a hyperbolic curve using equation 1.

$$\Delta A = \frac{(B_{\max} \bullet S)}{K_S + S} \quad (1)$$

Spectral binding experiments were also performed with flurbiprofen in the presence of 100 µM dapsone in a manner identical to that described above.

Spin State determination

Substrate-induced spin state changes with each of the CYP2C9 enzymes were measured as described above for spectral binding measurements. Spectral titrations with (*S*)-flurbiprofen and dapsone (concentrations were identical to those used for NMR experiments) were performed by adding equal volumes of either flurbiprofen, dapsone or flurbiprofen + dapsone to both sample and reference cuvettes followed by spectral scanning between 320 and 500 nm. Prior to scanning, the sample and reference cuvettes were allowed to equilibrate for 3 min with enzyme and substrate. Absolute spectra were then obtained to estimate the percent conversion of CYP2C9 from low spin to high spin using the relative low spin peak area [15]. Deconvolution of spectra was carried out with the multiple Gaussian curve fitting available in OriginPro (v7.5 OriginLab Corporation, Northampton, MA). The model was developed to include three components: a low spin component (~416–420 nm), a high spin component (~390–405 nm) and δ -bands (~360 nm). The amount of low spin heme iron is correlated to the low spin peak areas deconvoluted from samples of different P450 concentrations ($R^2 = 0.99$).

NMR sample preparation

Samples were prepared by a 50-fold dilution of the purified enzyme of interest into 50 mM potassium phosphate (pH 7.4), in D₂O, to reduce the concentration of glycerol and H₂O in the sample. Enzyme and substrate concentrations were based on the calculated K_S for each variant so that all enzymes present in the sample were saturated with substrate. For CYP2C9.1, 0.014

μM enzyme was used along with 145 μM flurbiprofen, 145 μM flurbiprofen and 100 μM dapsone, or 100 μM dapsone. For CYP2C9.2, 0.005 μM enzyme was used along with 50 μM flurbiprofen, 50 μM flurbiprofen and 100 μM dapsone, or 100 μM dapsone. For CYP2C9.3, 0.014 μM enzyme was used along with 140 μM flurbiprofen, 140 μM flurbiprofen and 100 μM dapsone, or 100 μM dapsone. For CYP2C9.5, 0.016 μM enzyme was used along with 160 μM flurbiprofen, 160 μM flurbiprofen and 100 μM dapsone, or 100 μM dapsone. All samples were made up in a final volume of 750 μL .

T_1 relaxation measurements

T_1 times of substrate protons were determined with the NMR instrument operating at 600.5 MHz, internally locked on the deuterium signal of the solvent. NMR spectra were acquired on a Varian Inova® 600 MHz NMR (Varian Instruments, Palo Alto, CA). The probe was maintained at 298° K for all experiments except for those involving temperature dependence. The Varian T_1 inversion-recovery sequence (d_1 -180- d_2 -90) was used along with presaturation of the residual HOD signal. The PW 90 was calibrated on each sample. Spectra were acquired for 12 τ (d_2) values ranging from 0.0125–25.6 s and a period of 10 T_1 was used between pulses (d_1). The Varian software routines were used to determine the T_1 times. Once the paramagnetic effect of the heme-iron on substrate protons was measured, CO was bubbled through the sample for 15 minutes, sodium dithionite was added and the sample allowed to equilibrate for 30 minutes then measured again, in order to determine the diamagnetic contribution to the T_1 relaxation times. Stability of the enzyme as well as the CO reduced complex was tested and both were found to be stable for the duration of the NMR acquisition time.

T_1 Temperature dependence

The validity of the T_1 measurements and the distances calculated between protons in flurbiprofen and dapsone is dependent on the substrates being in fast exchange. This can be demonstrated by conducting T_1 measurements over a range of temperatures [16]. Therefore, T_1 measurements were performed as described above at three different temperatures (283°, 298° and 310° K). Data were collected both in the absence ($1/T_1$, 2C9) and presence ($1/T_1$, 2C9+CO) of CO/sodium dithionite. To assure adequate diffusion of CO and mixing of dithionite, samples were removed from the NMR tube and placed in a test tube, CO bubbled into the sample and sodium dithionite added, then the sample was placed back into the NMR tube.

Distance calculations—In our previous work [12] on distances between heme and flurbiprofen/dapsone, estimates were calculated using the equation: $r = C[T_{1p} * \alpha_m * f(\tau_c)]^{1/6}$, where r is the distance and C is a constant that is a function of the metal, oxidation state, and whether it is low or high spin. In this case, Fe^{+3} is in the low spin state and thus the appropriate value for C is 539 [17]. However, we have since obtained spin-state data and this permits a more precise calculation. In particular, here distances were calculated from the Solomon-Bloembergen equation (equation 2) which can be written [18]:

$$\frac{1}{T_{1p}} = \alpha \frac{9.87 \times 10^{16} S(S+1)}{r^6} (\tau_c) \quad (2)$$

T_{1p} is the portion of $T_{1\text{obs}}$ due to paramagnetic effects alone and is given by $T_{1p}^{-1} = T_{1\text{obs}}(\text{Fe}^{+3})^{-1} - T_{1\text{obs}}(\text{Fe}^{+2})^{-1}$ assuming that all of the diamagnetic contribution is represented by $T_{1\text{obs}}(\text{Fe}^{+2})$ [16]. This assumption has been used in many similar studies and appears to be generally valid [19;20]. The correlation time (τ_c) for CYP2C9 has been previously reported ($2 \times 10^{-10} \text{ sec}^{-1}$) [19] and was used here.

The paramagnetic relaxation rate for a given resonance is the weighted average of individual equivalent nuclei that give rise to the resonance. The 2'/6' and 3'/5' protons of flurbiprofen are

equivalent and can be oriented with the aromatic ring bearing these protons parallel or perpendicular to the plane of the iron containing heme. When parallel,

$$\frac{1}{T_{1p, \text{apparent}}} = \frac{\frac{1}{T_{1p1}} + \frac{1}{T_{1p2}} + \dots + \frac{1}{T_{1pn}}}{n} \quad (3)$$

and $T_{1p, \text{apparent}} = (n \cdot 1/T_{1pn})/n$ or $1/T_{1pn}$ since $1/T_{1p1} = 1/T_{1p2} = \dots 1/T_{1pn}$. However, when perpendicular only one proton of each pair is near the heme plane and, due to the r^6 dependence, the contribution of the remote proton is negligible. Therefore, since $1/T_{1p1} \gg 1/T_{1p2}$, $T_{1p, \text{apparent}} = 1/2 T_{1p1}$.

The situation with dapsone is more complicated in that there are two possible parallel orientations (both rings centered over the heme (symmetrical and parallel) and only one ring parallel (unsymmetrical and parallel) and two perpendicular orientations (symmetrical/perpendicular and unsymmetrical/perpendicular). These orientations render the following sets of dapsone protons: 1) 2/2'/6/6' and 3/3'/5/5' (symmetrical/parallel), 2) 2/6, 3/5, 2'/6' and 3'/5' (unsymmetrical/parallel), 3) 2/2', 3/3' and 5/5', 6/6' (symmetrical/perpendicular), and 4) 2, 3, 5, 6, 2'/6', and 3'/5' (unsymmetrical/perpendicular). Set 1 can be calculated as for the parallel orientation for flurbiprofen, sets 2 and 3 can be calculated as for flurbiprofen in the perpendicular orientation. For the final set, only protons 2 and 3 (or equivalent sets, e.g. 2',3') are close to iron. The protons 5, 6, are sufficiently far from iron, due to the r^6 dependence, such that their contribution to the relaxation time is much smaller than 2 and 3. The corresponding protons 2', 3', 5', 6' are even more remote that their contribution can be neglected (i.e., $1/T_{1p1} \gg 1/T_{1p2} \gg 1/T_{1p3} \gg 1/T_{1p4}$ corresponding to 2, 6, 2', 6' or 3, 5, 3', 5', respectively). Thus, $1/T_{1p, \text{apparent}} = 1/4 T$.

$$\alpha = \frac{[EF] + [EFD]}{[F_0]} = \frac{\frac{[E_0]}{K_{DF}} + \frac{[E_0][D]}{K_{DF}K_{DFD}}}{1 + \frac{[F_0]}{K_{DF}} + \frac{[D_0]}{K_{DD}} + \frac{[F_0][D_0]}{K_{DF}F_{DDF}}}$$

The parameter α_m , the fractional binding coefficient, is equal to $[P450]/(K_S + [\text{substrate}])$ under conditions of fast exchange [16] when only flurbiprofen or dapsone is present. When both are present and under fast exchange conditions, α is calculated from equation 4.

Where E, F, and D are CYP2C9, flurbiprofen and dapsone, respectively and the equilibrium constants K are defined as shown in equation 5.

Finally, since the spin-state was found to be dependent upon substrate binding, the $S(S+1)$ term was approximated by equation 6, which effectively reduces to equation 7.

$$S(S+1) = f_{HS} S_{HS}(S_{HS}+1) + f_{LS} S_{LS}(S_{LS}+1) \quad (6)$$

$$S(S+1) = 8.75 f_{HS} + 0.75 f_{LS} \quad (7)$$

where $S_{HS} = 5/2$, $S_{LS} = 1/2$, and f_{HS} and f_{LS} refer to the fraction of the high and low spin iron, respectively.

Results

The chemical structures along with the proton numbering schemes used throughout for flurbiprofen and dapsone appear in Figure 1.

Determination of Spectral Binding Constants

The spectral binding constants (K_S) of the CYP2C9 variant forms for flurbiprofen in the absence and presence of dapsone were determined to calculate the proper enzyme and substrate concentrations to be used for the T_1 relaxation studies. Table 1 lists the K_S values obtained for flurbiprofen binding, in the absence and presence of dapsone, with CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5, respectively. The spectra obtained with CYP2C9.1 in the absence and presence of dapsone are presented in Figures 2A and 2B. All CYP2C9 enzymes gave a spectra characteristic of Type I binding and were qualitatively similar for all enzymes studied. At concentrations up to 100 μM , dapsone did not produce a binding spectra (either Type I or Type II), and thus did not interfere with assessment of flurbiprofen binding when mixed concurrently (data not shown). Binding constants obtained with CYP2C9.3 and CYP2C9.5 were approximately two-fold higher than observed with CYP2C9.1 and CYP2C9.2. Addition of dapsone resulted in a decrease in the binding constant in each of the enzymes (wild type and variant proteins).

Spin state

Percent low and high spin enzyme with no substrate, in the presence of the substrate flurbiprofen, in the presence of the effector dapsone and with both substrate and effector were determined (Table 2). To facilitate the NMR experiments and distance calculations, the concentration of substrate and effector were identical to those used in the T_1 studies. Each of the CYP2C9 enzymes, in the absence of substrate, existed in primarily the low spin state. Addition of flurbiprofen resulted in a 2–3 fold increase in the percent high spin enzyme in all cases. Furthermore, the addition of dapsone resulted in an additional doubling of the percent high spin enzyme in all cases, except CYP2C9.5 where the increase was much more modest. Addition of dapsone alone increased the percent high spin enzyme to approximately the same degree as did flurbiprofen alone.

Validation of Fast-Exchange Conditions

In order to ensure valid proton to heme distance estimates, the temperature dependence of the $T_{1\rho}$ for flurbiprofen and dapsone protons must behave in a temperature dependent manner in order to confirm that the molecules that come in contact with Fe^{3+} and those in the bulk solution are operating under fast-exchange conditions. The temperature dependence of $T_{1\rho}$ for flurbiprofen and dapsone protons for all CYP2C9 variants is shown in Figure 3. A positive linear plot of $1/T_{1\rho}$ versus $1/\text{Temperature}$ is indicative of fast-exchange conditions.

Determination of Proton-Heme Iron Distances

T_1 relaxation studies were conducted to determine the average substrate proton to heme distances for flurbiprofen in the absence and presence of dapsone with each of the enzyme proteins, CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5, respectively (Tables 3–4). The aromatic region of the ^1H NMR of flurbiprofen and dapsone is shown in Figure 4. There is little overlap of the resonances on which T_1 measurements were made. Addition of dapsone resulted in a decrease in the distance of the 4'-H (site of metabolism) from the heme iron in all proteins. T_1 -based distances were calculated for two orientations of flurbiprofen relative to the heme iron in which the ring bearing the 2'-6' protons is parallel or perpendicular to the plane of the heme ring system. Due to the symmetry of dapsone, four orientations are possible in which 1) both rings in dapsone are parallel to the heme plane and symmetrically disposed about the heme iron, 2) only one ring over and parallel to the heme ring system, and 3) two additional orientations where dapsone is rotated about its long axis by 90° such that the aromatic rings of dapsone are perpendicular to the heme plane. Distance calculations based on these orientations provide limits on proton-heme iron separations.

Discussion

It is becoming evident that not only are drug-drug interactions involving P450s dependent on substrate and effector but also can be allelic variant dependent. For example, genetic variant dependent activation of CYP2C9 by dapsone has been demonstrated, *in vitro* [9]. In each variant, the catalytic efficiency was increased due to both a reduction in K_m as well as an increase in V_m but to varying degrees, depending on the protein. In these previous studies, the efficiency (V_m/K_m) of flurbiprofen hydroxylation in the CYP2C9.3 variant was increased 4600% when co-incubated with dapsone, compared to flurbiprofen alone. CYP2C9.2, CYP2C9.5 and CYP2C9.1 also exhibited increases in efficiency of 3029%, 2100%, and 690%, respectively, due to co-incubation with dapsone. The reduction in flurbiprofen 4'-hydroxylation noted in the variants in the absence of dapsone and these striking differences in degree of activation suggest that the R144C, I359L, and D360E amino acid substitutions not only are key players in flurbiprofen 4' hydroxylation, but also in dapsone activation of flurbiprofen 4'-hydroxylation. However, full elucidation of this role remains unclear.

The substituted CYP2C9 residues resulting from genetic polymorphisms may change substrate and/or effector binding, affect folding of the protein, alter electron transfer from redox partners, or affect the efficiency of the enzyme through some other unknown mechanism(s). It has been hypothesized that differences in substrate proton to heme-iron distances among the CYP2C9 proteins might play a role in the previously observed differences in catalytic activity. Likewise, since the degree of dapsone activation of flurbiprofen metabolism differed among the CYP2C9 variants as compared to wild type protein, then perhaps these differences in activation could be explained by differences in the change of proton-heme distances due to the presence of dapsone would be observed among the proteins.

T_1 relaxation studies have been used successfully to estimate distances for codeine in CYP2D6 [20], diclofenac in CYP2C9 [19], caffeine in CYP1A2 [16], flurbiprofen and dapsone in CYP2C9 [12] and acetaminophen and caffeine in CYP3A4 [18]. The present study demonstrated that in all CYP2C9 proteins studied (CYP2C9.1, CYP2C9.2, CYP2C9.3 and CYP2C9.5) the flurbiprofen proton to heme iron distances, notably at the 4'-H which is the primary site of metabolism, though variable, did not directly correlate with previously observed differences in enzyme activity and correspondingly not directly with the amino acid substitutions in the variant proteins, either. These findings suggest that altered proton to heme-iron distances is not the only factor playing a role in the noted reductions in catalytic activity of the variant proteins [9].

In the presence of the effector molecule dapsone, the flurbiprofen protons and notably the 4'-H moved closer to the heme-iron in all the variants, though to varying degrees. Precedent exists for reduction in distances between substrate and heme-iron to cause a decrease in excess water formation, and thus an increase in enzyme efficiency [21]. Thus, it may be that this movement of the site of metabolism closer to the oxidizing species also plays a role in heteroactivation noted in the allelic variants of CYP2C9. However, in the present case, this, alone, cannot explain all of the observed differences. For example, the change in the heme-H4' distance upon addition of dapsone was 0.08 Å in CYP2C9.2 and activation was 3029% while the corresponding decrease for CYP2C9.5 was 0.44 Å and activation was 2100%. Likewise, the separation between heme-H4' cannot be the sole factor driving the differences. In the presence of dapsone, the heme-H4' separations for CYP2C9.1, CYP2C9.2 and CYP2C9.3 are identical within experimental error. In CYP2C9.5, this separation is significantly less (>0.40 Å closer than the other variants) and yet, for the former three, the range in activation by dapsone spans 4000% with the latter falling within this range (2100% activation). The lack of any apparent correlation between the heme-H4' separation and variant kinetics suggests that differences in degree of activation of the CYP2C9 variants by dapsone are not solely due to differential movement of substrate (flurbiprofen) within the CYP2C9 active site. It should be noted that changes in proton to heme-iron distances in the variant proteins by heteroactivators, such as dapsone, appear to be effector dependent since previous studies of CYP2C9.3 activation by benzbromarone did not observe changes in these distances [22].

The results obtained here with the wild-type protein (CYP2C9.1) are in agreement with our previous work [12] both in terms of qualitative effect (movement of flurbiprofen protons closer to the heme-iron) and quantitative effect (~0.9 Å shift in the H4' toward the heme-iron). Note that the absolute values for the distances between heme-H4' do differ but this is solely due to the different methods used to calculate T_1 values between our previous work and the current results (methods described in the experimental section). On a relative basis, there is no difference. Of note, the T_1 times in the presence of active P450 with Fe^{3+} for all flurbiprofen and dapsone protons were decreased compared to the CO dithionite-treated Fe^{2+} form of the P450. These findings would not be observed if flurbiprofen and dapsone were not both near the paramagnetic heme-iron and thus, it can be concluded that flurbiprofen and dapsone are binding simultaneously within the active site in each of the CYP2C9 variants, as has been

suggested for CYP2C9.1 [12]. Since dapsone also was oriented slightly closer to the heme-iron in the presence of flurbiprofen in all variants and generally tracks the changes observed for the 4'-H of flurbiprofen, these results also suggest that dapsone and flurbiprofen are directly interacting with one another in addition to interacting with the active site.

Not only does dapsone enhance enzyme efficiency and decrease the distance of flurbiprofen protons from the heme, but it also increases the affinity of the enzyme for flurbiprofen as demonstrated by the reduction in spectrally determined binding constants (K_S) for all variants. Hutzler et al. observed a similar decrease in K_S with flurbiprofen in the presence of dapsone in CYP2C9.1, changing from 14 μM (no dapsone) to 2 μM (dapsone present) [14]. Interestingly, CYP2C9.1 and CYP2C9.2 displayed similar binding constants for flurbiprofen alone and with dapsone present. CYP2C9.2 typically exhibits decreased metabolism of substrates, mostly through a decrease in V_m , with little or no effect on K_m [9;23]. CYP2C9.3 and CYP2C9.5 exhibited similar K_S values, suggesting similar binding affinities, but approximately double that observed with CYP2C9.1 and CYP2C9.2 enzymes. The reduction in metabolism exhibited with these two variants is usually associated with a decrease in V_m , as well as an increase in K_m [1;9;23;24]. Also, the decreases in K_S due to the presence of dapsone are of greater magnitude when studied in the CYP2C9 variants compared to the wild type enzyme, correlating reasonably well with the percent activation of flurbiprofen metabolism noted in each of the variants and the wild type enzyme [9], i.e., the greatest changes in K_S noted in the variants exhibiting the greatest percent activation.

The substitution of a negatively charged cysteine in place of a positively charged arginine in CYP2C9.2 (R144C) has been associated with reduced substrate metabolism both *in vitro* and *in vivo*, primarily by reducing V_m , with little effect on K_m [9;23]. It was previously suggested that the reduction in substrate turnover is likely due to alterations in the association of CYP2C9.2 with P450 reductase; the interaction of these two proteins is essential for electron transport necessary for turnover of substrates [6]. In the crystal structure with flurbiprofen bound, position 144 is in the D helix which is on the surface of the protein [8], though it is unknown whether this area is involved in P450 reductase binding. More recently, through a series of stoichiometric studies with CYP2C9 variants [5], it has been suggested that other mechanisms are involved in the reduced activity of the CYP2C9.2 protein. These studies demonstrated that the P450 cycle is actually triggered slightly more per second in the CYP2C9.2 protein as compared to the CYP2C9.1 protein; a finding counter to what would be expected if diminished electron transport from P450 reductase were occurring. Furthermore, these same studies also demonstrated a similar K_d value for the binding of P450 reductase to CYP2C9.2 as compared to the wild type enzyme. In the current study, the presence of dapsone decreased the distance of flurbiprofen protons from the heme-iron and reduced the K_S for flurbiprofen binding suggesting that this amino acid substitution may have a more global impact on either the conformation of the enzyme or the functioning of the catalytic cycle; a finding in congruence with the more recent results from stoichiometry studies [5].

The CYP2C9.3 variant has a leucine residue in place of an isoleucine residue at amino acid position 359 and the CYP2C9.5 variant contains a glutamate in place of an aspartate at position 360 [1;24]. Although CYP2C9.3 and CYP2C9.5 have modest amino acid substitutions, they exhibit substantially reduced substrate turnover compared to the wild type enzyme [1;24]. Both positions 359 and 360 are located in substrate recognition site (SRS) 5 and thus, have the potential to play a role in substrate binding [25]. More recently, published crystal structures of CYP2C9 suggest that though they are located in the active site, these amino acid positions may not have direct contact with substrate [7;8]. It has been postulated that the reduced activity noted in the CYP2C9 polymorphic enzymes may be due to these amino acid changes causing a disruption of the water matrix within the enzyme active site, with a corresponding alteration in P450 cycle coupling [5]. The K_S values observed with the .3 and .5 proteins are only modestly

different (~2-fold higher) from the wild-type protein, despite marked differences in K_m . This suggests that a change in k_{cat} (a component of K_m , wherein $K_m = \frac{k_{-1} + k_{cat}}{k_1}$ and $K_s = \frac{k_{-1}}{k_1}$) is more likely the cause of the noted reductions in substrate metabolism in the .3 and .5 proteins, rather than changes in substrate binding (K_s). That dapson co-incubation results in substrate moving closer to the heme-iron with subsequent activation of all the CYP2C9 forms, together with the differential effects on K_s and K_m suggest that enzyme activation mechanisms are multi-factorial and involve changes in binding affinity, substrate orientation and subsequently, modification of P450 catalytic cycle efficiency. It is tempting to speculate that one of the reasons for the greater activation noted in the allelic variants is simply because the polymorphic enzymes have a much lower baseline activity and thus, more room for improvement in efficiency. In contrast, the wild type protein may be operating at much closer to maximum efficiency and thus, have less percent capacity for improvement in efficiency.

Alternatively, or perhaps in addition, interaction between substrate and effector may play a role. CYP2C9.2, CYP2C9.3 and CYP2C9.5 metabolize flurbiprofen at 70%, 4% and 10%, respectively, of the rate of CYP2C9.1 [9]. In the presence of dapson, the activation observed in flurbiprofen metabolism by CYP2C9.1, CYP2C9.2, CYP2C9.3 and CYP2C9.5 is increased by 690%, 3029%, 4600%, and 2100%, respectively. In addition, the binding constant for CYP2C9.2 is approximately three times greater than the either the wild type or other variants. Taking the reduction in rate due to variant, observed increase in each variant due to the presence of dapson, and normalizing for the binding constant gives activation increases of 690%, 707%, 184%, and 210%. Thus, the activity of the CYP2C9.2 variant is restored to the level of CYP2C9.1 and in CYP2C9.3 and CYP2C9.5 is about one-third the level of the wild-type. This suggests that the changes in structure caused by the mutations resulting in these variants may be 'undone' by the presence of both flurbiprofen and dapson but is not possible for either flurbiprofen or dapson to achieve alone. By inference, then, this suggests that flurbiprofen and dapson are behaving as a single entity, suggesting that flurbiprofen and dapson strongly interact with one another.

In summary, flurbiprofen and dapson were shown to be oriented near the heme-iron within the active sites of the CYP2C9 allelic variants, with similar proton to heme-iron distances noted irrespective of the variant enzyme studied. The distances of flurbiprofen from the heme-iron were reduced when dapson was present and to a roughly equal extent in each of the CYP2C9 variants. Spectral binding studies revealed that the presence of dapson increased the affinity (decreased K_s) of the CYP2C9 variants for flurbiprofen to a greater extent, as well. These results demonstrate that both changes in substrate orientation and distance to the heme-iron, as well as changes in substrate affinity, contribute to the mechanism(s) of activation in CYP2C9 variants. In addition, the results provide additional evidence that drug-drug interactions may be allelic variant dependent and care must be taken in order to account for these differences in pre-clinical drug development studies.

Acknowledgements

This work was funded by NIH Grants #GM063215 and GM069753 to TST. The authors would also like to acknowledge the assistance of Dr. Chuck Locuson and Dr. Murali Subramanian in conducting the spin state determinations.

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Figure 1. Structures of flurbiprofen and dapsone
Numbering schemes used in the text are shown.

Figure 2. Flurbiprofen spectral binding determined in CYP2C9.1 by UV/Vis spectroscopy
 A Type I spectrum is evident with the peak of absorbance at ~390 nm and the trough at ~420 nm for both Panel A. - flurbiprofen alone and Panel B. - flurbiprofen and 100 μM dapsone. Insets are the respective binding curves and K_s values derived from fitting of the data to equation 1. Though not shown, spectral binding curves for CYP2C9.2, CYP2C9.3 and CYP2C9.5 were qualitatively similar.

Figure 3. Temperature dependence of $T_{1\rho}$ of flurbiprofen and dapsone protons in CYP2C9 allelic variants

A positive linear slope is indicative of fast-exchange conditions. Legend: Flurbiprofen H-2 (●), H-5 (○), H-6 (▼), H-2'/6' (▽), H-3'/5' (■), H-4' (□), CH₃ (◆). Dapsone H-2/2'/6/6' (◇), H-3/3'/5/5' (▲). Panel A. CYP2C9.1, Panel B. CYP2C9.2, Panel C. CYP2C9.3, Panel D. CYP2C9.5

Figure 4. NMR of the aromatic region of flurbiprofen and dapsone obtained under conditions used for T_1 measurements

Protons due to dapsone are indicated with DAP. The remaining protons correspond to flurbiprofen. Chemical shift assignments are shown. The numbering scheme used is the same as shown in Figure 1.

Table 1

Spectrally Determined Binding Constants for Flurbiprofen, in the Absence and Presence of Dapsone, Obtained with CYP2C9.1, CYP2C9.2, CYP2C9.3 and CYP2C9.5 Enzymes.

Enzyme	No Dapsone K_S (μ M)	+ Dapsone K_S (μ M)
CYP2C9.1	7.5	2.8
CYP2C9.2	4.5	1
CYP2C9.3	13.8	3
CYP2C9.5	16	3

Table 2

Percent low and high spin CYP2C9 enzyme in the presence of flurbiprofen and/or dapsone

Enzyme (+ substrate)	% Low Spin	% High Spin
CYP2C9.1 (no substrate)	96.0	4.00
+ Flurbiprofen	84.3	15.7
+ Flurbiprofen and Dapsone	73.2	26.8
+ Dapsone	88.7	11.3
CYP2C9.2 (no substrate)	94.8	5.24
+ Flurbiprofen	88.8	11.2
+ Flurbiprofen and Dapsone	74.3	25.7
+ Dapsone	86.1	13.9
CYP2C9.3 (no substrate)	96.9	3.14
+ Flurbiprofen	87.1	12.9
+ Flurbiprofen and Dapsone	75.5	24.5
+ Dapsone	90.8	9.19
CYP2C9.5 (no substrate)	92.9	7.15
+ Flurbiprofen	89.8	10.2
+ Flurbiprofen and Dapsone	86.5	13.5
+ Dapsone	86.4	13.6

Table 3
T₁ Relaxation Times of Protons in Flurbiprofen and Dapsone in CYP2C9 Variants.^a

Compound ^d	Proton Resonance ^e	CYP2C9.1 ^b			CYP2C9.2 ^c		
		Flurbiprofen or Dapsone T ₁ 2C9	Flurbiprofen and Dapsone T ₁ 2C9+CO	Flurbiprofen and Dapsone T ₁ 2C9	Flurbiprofen or Dapsone T ₁ 2C9+CO	Flurbiprofen and Dapsone T ₁ 2C9	Flurbiprofen and Dapsone T ₁ 2C9+CO
Flurbiprofen	CH ₃	0.71 (0.00)	0.73 (0.01)	0.70 (0.01)	0.72 (0.01)	0.67 (0.01)	0.69 (0.01)
	2	2.67 (0.06)	3.15 (0.08)	2.54 (0.06)	3.06 (0.07)	2.50 (0.10)	2.99 (0.22)
	5	2.22 (0.05)	2.43 (0.05)	2.18 (0.04)	2.49 (0.04)	2.18 (0.12)	2.20 (0.23)
	6	1.95 (0.03)	2.20 (0.04)	1.98 (0.03)	2.19 (0.03)	1.96 (0.10)	1.90 (0.13)
	2',6'	2.46 (0.03)	2.83 (0.03)	2.47 (0.02)	2.85 (0.03)	2.44 (0.09)	2.94 (0.11)
	3',5'	2.49 (0.03)	2.85 (0.03)	2.46 (0.03)	2.86 (0.04)	2.27 (0.07)	2.81 (0.12)
Dapsone	4'	3.87 (0.16)	4.67 (0.19)	3.59 (0.07)	4.86 (0.19)	3.25 (0.26)	4.61 (0.57)
	2,2',6,6'	3.78 (0.02)	4.83 (0.06)	3.79 (0.02)	4.87 (0.03)	3.89 (0.03)	4.58 (0.06)
	3,3',5,5'	4.27 (0.04)	5.31 (0.08)	4.28 (0.03)	5.26 (0.05)	4.34 (0.05)	5.31 (0.10)

^aErrors for measurements were < 10%.

^b[P450 2C9.1] = 0.016 μM

^c[P450 2C9.2] = 0.005 μM

^dCYP2C9.1 [Flurbiprofen] = 145 μM; [Dapsone] = 100 μM, CYP2C9.2 [Flurbiprofen] = 50 μM; [Dapsone] = 100 μM, CYP2C9.3 [Flurbiprofen] = 140 μM; [Dapsone] = 100 μM, CYP2C9.5 [Flurbiprofen] = 160 μM; [Dapsone] = 100 μM.

^eSee Figure 1 for numbering scheme of the protons for flurbiprofen and dapsone. T₁ times for the HC-CO₂H proton could not be accurately determined due to interference from the residual glycerol resonances.

^f[P450 2C9.3] = 0.014 μM

^g[P450 2C9.5] = 0.016 μM

Table 4
Distances of Protons in Flurbiprofen and Dapsone in CYP2C9 Variants.^a

Compound	Proton Resonance ^b	r (Å) ^c	r (Å) ^d	r (Å) ^e	r (Å) ^f	r (Å) ^g
Flurbiprofen	CH ₃	6.77 (0.06)	7.61 (0.06)	5.71 (0.10)	6.42 (0.11)	7.34 (0.12)
	2	6.35 (0.01)	7.13 (0.02)	6.09 (0.37)	6.84 (0.42)	7.25 (0.22)
	5	6.77 (0.15)	7.60 (0.18)	5.66 (0.46)	6.35 (0.52)	7.05 (0.19)
	6	6.33 (0.10)	7.10 (0.11)	6.05 (0.34)	6.80 (0.38)	6.98 (0.16)
	2',6'	6.43 (0.08)	7.21 (0.10)	6.07 (0.22)	6.81 (0.25)	7.07 (0.13)
	3',5'	6.48 (0.09)	7.27 (0.10)	5.54 (0.22)	6.22 (0.12)	7.02 (0.14)
Dapsone	4'	6.62 (0.27)	7.44 (0.30)	5.76 (0.45)	6.47 (0.51)	7.06 (0.27)
	2,2',6,6'	5.84 (0.07) ⁱ	6.55 (0.08) ^k	6.28 (0.09)	7.05 (0.10)	7.98 (0.10)
	3,3',5,5'	6.55 (0.08) ^j	7.36 (0.09) ^l	7.05 (0.10)	7.91 (0.11)	8.96 (0.12)
		6.06 (0.01) ⁱ	6.81 (0.11) ^k	6.31 (0.10)	7.08 (0.11)	7.94 (0.13)
		6.81 (0.11) ^j	7.64 (0.12) ^l	7.08 (0.11)	7.95 (0.13)	8.91 (0.14)
	CH ₃	6.21 (0.06)	6.97 (0.06)	5.71 (0.12)	6.40 (0.13)	6.85 (0.14)
Flurbiprofen and Dapsone	2	5.69 (0.06)	6.39 (0.12)	5.86 (0.49)	6.58 (0.55)	6.45 (0.18)
	5	5.84 (0.29)	6.56 (0.33)	5.82 (0.58)	6.54 (0.65)	7.19 (0.24)
	6	6.00 (0.21)	6.74 (0.24)	6.79 (0.48)	7.62 (0.53)	6.84 (0.23)
	2',6'	5.90 (0.18)	6.62 (0.20)	5.68 (0.20)	6.37 (0.23)	6.04 (0.11)
	3',5'	5.85 (0.18)	6.56 (0.20)	5.70 (0.37)	6.49 (0.42)	5.81 (0.13)
	4'	5.61 (0.23)	6.29 (0.26)	5.68 (0.48)	6.37 (0.58)	5.66 (0.27)
	2,2',6,6'	5.82 (0.04) ⁱ	6.53 (0.05) ^k	6.17 (0.08)	6.92 (0.10)	6.02 (0.08)
		6.53 (0.05) ^j	7.33 (0.06) ^l	6.92 (0.10)	7.77 (0.11)	6.76 (0.09)
	3,3',5,5'	6.11 (0.06) ⁱ	6.86 (0.07) ^k	6.07 (0.08)	6.81 (0.13)	6.80 (0.13)
		6.86 (0.07) ^j	7.70 (0.08) ^l	6.81 (0.13)	7.65 (0.15)	7.64 (0.15)

^aErrors for measurements were < 10%.

^bSee Figure 1 for numbering scheme of the protons for flurbiprofen and dapsone. T₁ times for the HC-CO₂H proton could not be accurately determined due to interference from the residual glycerol resonances.

^c[P450 2C9.1] = 0.016 μM, [Flurbiprofen] = 160 μM; [Dapsone] = 100 μM. αM = [P450]/(K_S+[substrate]), K_S (flurbiprofen) = 16.0 μM, αM (flurbiprofen) = 9.15×10⁻⁵, K_S (dapsone) = 100 μM (not obtainable by UV spectroscopy, assumed K_S=K_M), αM (Dapsone) = 7.00×10⁻⁵, αM (flurbiprofen and Dapsone) = 3.72×10⁻⁵, S(S+1) = 2.07 (flurbiprofen), 1.65 (Dapsone), 2.07 (flurbiprofen +Dapsone).

^d[P450 2C9.2] = 0.005 μM, [Flurbiprofen] = 50 μM; [Dapsone] = 100 μM. αM = [P450]/(K_S+[substrate]), K_S (flurbiprofen) = 4.5 μM, αM (flurbiprofen) = 9.09×10⁻⁵, K_S (dapsone) = 100 μM (not obtainable by UV spectroscopy, assumed K_S=K_M), αM (Dapsone) = 7.00×10⁻⁵, αM (flurbiprofen and Dapsone) = 1.96×10⁻⁵, S(S+1) = 1.64 (flurbiprofen), 1.17 (Dapsone), 2.81 (flurbiprofen +Dapsone).

^e[P450 2C9.2] = 0.014 μM, [Flurbiprofen] = 140 μM; [Dapsone] = 100 μM. αM = [P450]/(K_S+[substrate]), K_S (flurbiprofen) = 14 μM, αM (flurbiprofen) = 9.09×10⁻⁵, K_S (dapsone) = 100 μM (not obtainable by UV spectroscopy, assumed K_S=K_M), αM (Dapsone) = 7.00×10⁻⁵, αM (flurbiprofen and Dapsone) = 3.95×10⁻⁵, S(S+1) = 1.78 (flurbiprofen), 1.00 (Dapsone), 2.71 (flurbiprofen +Dapsone).

$f_{[P450\ 2C9.2]} = 0.016\ \mu\text{M}$, $[Flurbiprofen] = 160\ \mu\text{M}$; $[Dapsone] = 100\ \mu\text{M}$. $\alpha_M = [P450]/(K_S + [\text{substrate}])$. K_S (flurbiprofen) = $16\ \mu\text{M}$. α_M (Flurbiprofen) = 9.09×10^{-5} , K_S (dapsone) = $100\ \mu\text{M}$ (not obtainable by UV spectroscopy, assumed $K_S = K_M$). α_M (Dapsone) = 7.00×10^{-5} , α_M (Flurbiprofen and Dapsone) = 3.72×10^{-5} , $S(S+1) = 1.56$ (Flurbiprofen), 1.83 (Flurbiprofen + Dapsone).

$g_{\tau(())} = [9.87 \times 10^{16} * T_{1P} * \alpha_M * S(S+1) * \tau_C]^{1/6}$, $\tau_C = 2 \times 10^{-10}$ [18] and corresponds to the parallel binding orientation of flurbiprofen.

$h_{\tau(')} = [9.87 \times 10^{16} * 2T_{1P} * \alpha_M * S(S+1) * \tau_C]^{1/6}$, $\tau_C = 2 \times 10^{-10}$ [18] and corresponds to the perpendicular binding orientation of flurbiprofen.

$i_{\tau(())} = [9.87 \times 10^{16} * T_{1P} * \alpha_M * S(S+1) * \tau_C]^{1/6}$, $\tau_C = 2 \times 10^{-10}$ [18] and corresponds to the symmetrical/parallel binding orientation of dapsone.

$j_{\tau(())} = [9.87 \times 10^{16} * 2T_{1P} * \alpha_M * S(S+1) * \tau_C]^{1/6}$, $\tau_C = 2 \times 10^{-10}$ [18] and corresponds to the unsymmetrical/parallel binding orientation of dapsone.

$k_{\tau(())} = [9.87 \times 10^{16} * 2T_{1P} * \alpha_M * S(S+1) * \tau_C]^{1/6}$, $\tau_C = 2 \times 10^{-10}$ [18] and corresponds to the symmetrical/perpendicular binding orientation of dapsone.

$i_{\tau(())} = [9.87 \times 10^{16} * 4T_{1P} * \alpha_M * S(S+1) * \tau_C]^{1/6}$, $\tau_C = 2 \times 10^{-10}$ [18] and corresponds to the unsymmetrical/perpendicular binding orientation of dapsone.