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Differential activation of CYP2C9 variants by dapsone

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Abstract

Studies have shown that CYP2C9.1 mediated metabolism of flurbiprofen or naproxen is activated by co-incubation with dapsone. However, dapsone activation has not been examined in the known variant forms of CYP2C9. Six concentrations of flurbiprofen (2–300 μM) or naproxen (10–1800 μM) were co-incubated with six concentrations of dapsone (0–100 μM) and with reconstituted, purified CYP2C9.1, CYP2C9.2 (R144C), CYP2C9.3 (I359L), or CYP2C9.5 (D360E), in order to assess degrees of activation. Dapsone increased the efficiency (V_m/K_m) of flurbiprofen 4′-hydroxylation by CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5 by 8-, 31-, 47-, and 22-fold, respectively. In similar experiments using the substrate naproxen, dapsone increased the efficiency of naproxen demethylation 7-, 15-, 13-, and 22-fold, in CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5, respectively. Also, dapsone normalized naproxen's kinetic profile from biphasic (CYP2C9.1 and CYP2C9.2) or linear (CYP2C9.3 and CYP2C9.5) to hyperbolic for all variant forms. Thus, amino acid substitutions of CYP2C9 variants affect the degree of dapsone activation in a genotype-dependent fashion. Furthermore, the degree of effect noted across variants appeared to be dependent on the substrate studied.

Keywords: CYP2C9; Flurbiprofen; Naproxen; Dapsone; Allelic variants; Activation

1. Introduction

Differences in metabolism among variants of cytochromes P450 are becoming increasingly important as knowledge is being gained about the in-vitro and in-vivo consequences of the single nucleotide polymorphisms that result in amino acid substitutions. One polymorphic P450 of importance is CYP2C9, because it is involved in the metabolism of a wide range of substrates, including *S*-warfarin, phenytoin, tolbutamide, as well as a number of NSAIDS [18,22,24,28,29,33]. When assessing the effects of CYP2C9 variants, the substrates of most concern are the low therapeutic index drugs, *S*-warfarin and phenytoin, because individuals expressing a variant may exhibit reduced clearance of these agents [4,8,23].

Five coding-region variants and a sixth null variant of CYP2C9 have been characterized to date, including *CYP2C9*1* (wild type), *CYP2C9*2* (R144C), *CYP2C9*3* (I359L), *CYP2C9*4* (I359T), *CYP2C9*5* (D360E), and *CYP2C9*6* (818delA). Of these, the most well characterized are CYP2C9.1, CYP2C9.2, and CYP2C9.3. Compared to individuals with the wild type enzyme, individuals expressing the CYP2C9.2 and CYP2C9.3 variants, found mostly in the Caucasian population, exhibit reduced metabolism of a number of substrates both in vitro and in vivo [7,9,25,26,31]. The CYP2C9.2 variant appears to reduce the V_m of substrate turnover but has little effect on K_m [31]. In subjects expressing the CYP2C9.3 variant, K_m is increased for all studied substrates whereas V_m is decreased [26]. Limited information is available concerning the rare variant, CYP2C9.4, which was found in a Japanese epileptic patient requiring a lower than normal dose of phenytoin, however in-vitro studies have shown it to behave similarly to CYP2C9.3 [14,15]. CYP2C9.5, which has been shown to be present in the African-American and African populations, has been associated with reductions in in-vitro intrinsic clearance of a number of substrates, including lauric acid, *S*-warfarin, diclofenac, flurbiprofen, naproxen, and piroxicam [3,27,32].

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However, in vivo consequences of this amino acid change are unknown at present. The most recently described allelic variant, *CYP2C9*6*, was identified in an African-American subject exhibiting phenytoin toxicity [16]. Since this protein is truncated as a result of a premature stop codon and is therefore lacking a heme-binding region, it is inactive.

Dapsone has been shown to activate the metabolism of flurbiprofen, naproxen, and piroxicam by wild-type CYP2C9.1 [11,12,17]. This activation results in a decrease in the K_m and an increase in the V_m of the CYP2C9 enzyme causing an increase in efficiency of the enzyme and substrate turnover. It has been proposed that the activator must bind simultaneously within the enzyme active site to elicit its effect [17]. Because amino acid changes in key residues, such as those found in the variants of CYP2C9, alter rates of metabolism, these substitutions may also affect the degree of activation observed. The degree of dapsone mediated activation of CYP2C9 variants has yet to be assessed. Using flurbiprofen and naproxen as probe substrates, the effects of dapsone on CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5 mediated metabolism were studied to determine whether the degree of enzyme activation is dependent on the enzyme variant being studied.

2. Materials and methods

2.1. Chemicals and reagents

(*S*)-Flurbiprofen, 4′-hydroxyflurbiprofen, and 2-fluoro 4-biphenyl acetic acid were gifts from Pharmacia Corporation. (*S*)-Naproxen and desmethylnaproxen were gifts from Syntex Laboratories. Dapsone, NADPH, and dilauroylphosphatidylcholine were purchased from Sigma–Aldrich. Acetonitrile and potassium phosphate were purchased from Fisher Scientific. Human P450 oxidoreductase and human cytochrome b_5 were purchased from Pan Vera LLC. All other chemicals were purchased from commercial sources and were of the highest purity available.

2.2. Enzyme construction and expression

Mutagenesis and expression vector construction of the wild type enzyme (CYP2C9.1), the R144C (CYP2C9.2), I359L (CYP2C9.3), and the D360E (CYP2C9.5) variants were carried out according to previously established methods [3,7]. Proteins were expressed from recombinant baculoviruses in *Trichoplusia ni* cells and purified as described previously [3,7].

2.3. In vitro reaction conditions

Metabolic incubations were carried out according to the methods of Tracy et al. [27]. Incubation mixtures contained 5–20 pmol of purified enzyme, NADPH reductase, and cytochrome b_5 in a 1:2:1 ratio, reconstituted with dilauroylphosphatidylcholine vesicles extruded through a 200 nm pore sized membrane. Six concentrations of substrate, either 2–300 μM (*S*)-flurbiprofen or 10–1800 μM (*S*)-naproxen, were incubated with six concentrations of dapsone, 0–100 μM, for 20 min at 37 °C in 50 mM potassium phosphate buffer, pH 7.4 in a final volume of 200 μl. The reactions were initiated by the addition of 1 mM NADPH after a 3 min preincubation. Reactions were quenched by the addition of 200 μl acetonitrile containing internal standard, 180 ng/ml of 2-fluoro-4-biphenylacetic acid for (*S*)-flurbiprofen or 500 ng/ml of 2-fluoro-4-biphenylacetic acid for (*S*)-naproxen. After quenching, 40 μl of half strength H_3PO_4 was added to the reaction mixtures for both substrates. Samples were then centrifuged at 10,000 rpm for 4 min, placed into autosampler vials, and 5–50 μl injected onto the HPLC system.

2.4. HPLC analysis

HPLC analysis was conducted as described previously [27]. The HPLC system consisted of a Waters Model 501 pump module, a Waters Model 717 autosampler, and a Waters Model 470 fluorescence detector. For both metabolites, the mobile phase was pumped through a Brownlee Spheri-5 C_{18} 4.6 mm × 100 mm column at 1 ml/min. For quantification of 4′-hydroxyflurbiprofen, the detector was set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm, and the mobile phase consisted of 45:55 acetonitrile:20 mM potassium phosphate, pH 3.0. The retention times for 4′-hydroxyflurbiprofen and the internal standard were approximately 2.6 and 5.6 min, respectively. For quantification of desmethylnaproxen, the detector was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm, and the mobile phase consisted of 40:60 acetonitrile:20 mM potassium phosphate, pH 3.0. The retention times for desmethylnaproxen and the internal standard were approximately 2.3 and 9.6 min, respectively.

2.5. Data analysis

Kinetic parameters for the substrates were estimated by nonlinear regression analysis, using Sigma Plot 7.0. Data were fit to the Michaelis–Menten equation:

$$v = \frac{V_m \times [S]}{K_m + [S]}, \quad (1)$$

a linear equation:

$$v = (CL_{int}) \times [S], \quad (2)$$

or a biphasic equation:

$$v = \frac{(V_{m1} \times [S])(CL_{int} \times [S]^2)}{K_m + [S]}, \quad (3)$$

in addition to a two-site model [11,17].

$$v = \frac{V_m \times [S]}{[K_m(1 + ([B]/K_B))/(1 + (\beta[B]/\alpha K_B))] + [S]\{(1 + ([B]/\alpha K_B))/(1 + (\beta[B]/\alpha K_B))\}} \quad (4)$$

Appropriateness of the fits and the best-fitting model (among Eqs. (1)–(3)) were determined by examination and comparison of the residuals, residual sum of squares, coefficients of determination and F values.

3. Results

Fig. 1 depicts the three-dimensional fits of the data to the two-site model (Eq. (4)) for dapsone activation of flurbiprofen 4′-hydroxylation by CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5. Differences in the degree of activation by dapsone among the four variants tested are evident from the fits of the data. The 3-D surface plots for CYP2C9.1 and CYP2C9.2 are similar in that they are nearing maximal activation near 50 μM dapsone, however, the surface plot slopes in the range of 0–50 μM dapsone differ. In contrast, CYP2C9.3 and CYP2C9.5 differ from the wild type and CYP2C9.2 variants in that a maximum activation of flurbiprofen 4′-hydroxylation does not appear to have been achieved at 100 μM dapsone concentrations as the slope of the surface plots along the dapsone concentration axis continues to rise without reaching a plateau. Five parameters can be derived from the two-site model including, the V_m and K_m for flurbiprofen 4′-hydroxylation

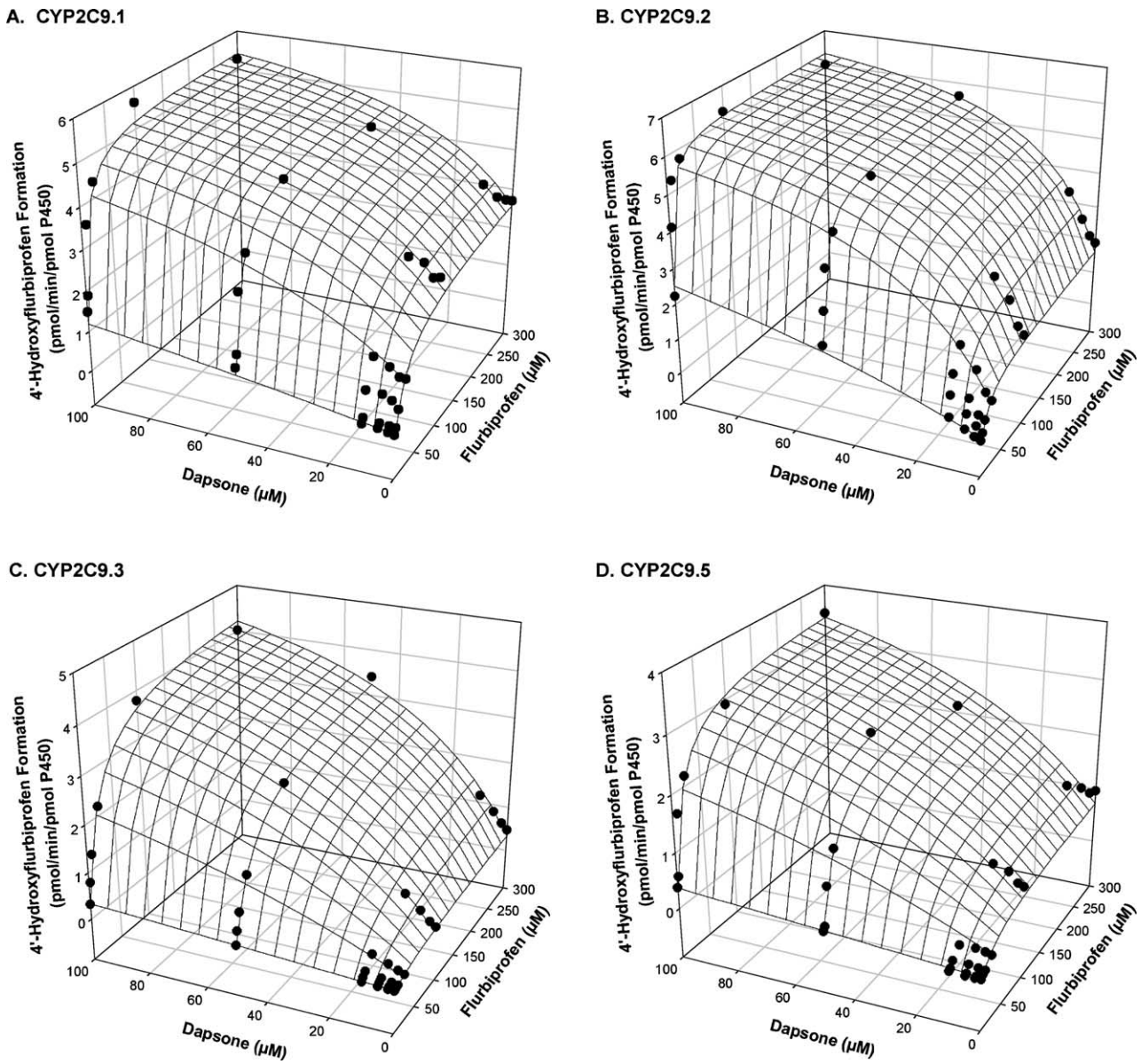

Fig. 1. Dapsone activation of flurbiprofen 4′-hydroxylation in (A) CYP2C9.1, (B) CYP2C9.2, (C) CYP2C9.3, and (D) CYP2C9.5. Scaling of each plot differs to permit better visualization of degree of activation. Data are presented as mean of duplicate determinations, with deviations between determinations <15% in all cases.

Table 1
Kinetic parameter estimates for dapsone activation of flurbiprofen 4′-hydroxylation[a]

Variant	V_m (pmol/min/pmol P450)	K_m (μM)	K_B (μM)	α	β	R^2
CYP2C9.1	2.67 (0.20)	23.5 (5.07)	178.30 (171.8)	0.16 (0.12)	2.38 (0.27)	0.959
CYP2C9.2	1.51 (0.12)	12.1 (2.09)	132.90 (61.1)	0.16 (0.07)	4.96 (0.42)	0.986
CYP2C9.3	0.41 (0.06)	70.0 (12.7)	410.10 (305.4)	0.11 (0.08)	15.60 (2.21)	0.995
CYP2C9.5	0.68 (0.13)	25.3 (11.4)	95.46 (92.3)	0.69 (0.70)	8.35 (2.22)	0.945

[a] Data fit to two-site model (Eq. (4)). Parameter estimates are reported as the estimate (standard error of the estimate) resulting from nonlinear regression of the mean of duplicate determinations (<15% variation in all cases). K_B, the binding constant for the effector; α, the change in K_m resulting from effector binding; and β, the change in V_m resulting from effector binding. An α value <1 indicates a decrease in K_m. A β value >1 indicates an increase in V_m.

in the absence of dapsone, an effector binding constant (K_B), the change in K_m as a result of effector binding (α), as well as the change in V_m as a result of effector binding (β). An α value <1 is indicative of a decrease in K_m due to the presence of effector and a β value >1 indicates an increase in V_m due to the presence of effector. Dapsone is the effector in this case. Kinetic parameter estimates for fits of the data to Eq. (4) (two-site model) are presented in Table 1. For all CYP2C9 variant forms tested, dapsone increased the metabolism of flurbiprofen by reducing the K_m ($\alpha < 1$) and increasing the V_m ($\beta > 1$). For comparison purposes, these data were also fit to the Michaelis–Menten equation at each of the six concentration of dapsone. The Michaelis–Menten kinetic parameter estimates are listed in Table 2. In the absence of dapsone, CYP2C9.1 exhibited the highest activity of flurbiprofen 4′-hydroxylation, followed by CYP2C9.2, CYP2C9.5, and CYP2C9.3, which is in agreement with the two-site model fits (Table 1). In CYP2C9.1, the V_m was increased roughly two-fold and the K_m decreased four-fold in the presence of 100 μM dapsone as compared to the absence of dapsone. Similar trends were noted for the other variants, although to varying degrees. Fold differences in increase of enzymatic efficiency (V_m/K_m) toward flurbiprofen 4′-hydroxylation in the presence of dapsone among the CYP2C9 variants are presented in Table 3.

Naproxen demethylation was also activated by the presence of dapsone in all of the CYP2C9 variants tested. Surface plots representing fits of the data for naproxen demethylation in the presence of dapsone to the two-site model (Eq. (4)) are presented in Fig. 2. Again, differences in the 3-D surfaces are evident among the variants. The velocities of CYP2C9.1 and CYP2C9.2 mediated naproxen demethylation appear to be reaching maximal activation near 50 μM dapsone concentrations with differing slopes up to that point. On the other hand, the 3-D surface plots for CYP2C9.3 and CYP2C9.5 do not reach maximal activation at 100 μM dapsone as evidenced by a lack of plateau. Kinetic parameter estimates based on these two-site model fits are presented in Table 4. The estimated α parameters are <1 and β values >1 in all CYP2C9 variant forms tested, suggesting a decrease in K_m and increase in V_m for naproxen metabolism due to the presence of dapsone in the incubation mixture. Because the kinetic profiles for naproxen demethylation transformed from either biphasic (CYP2C9.1 and CYP2C9.2) or linear (CYP2C9.3 and CYP2C9.5) to a hyperbolic form, parameter estimates with the best-fitting equation (biphasic (Eq. (3)), linear (Eq. (2)) or hyperbolic (Eq. (1))) were calculated and are presented in Table 5. In the cases of CYP2C9.1 in the presence of 0–5 μM dapsone and CYP2C9.2 in the presence of 0–2 μM dapsone, the resulting data were best fit to a biphasic equation, resulting in estimates of V_{m1} and K_{m1} (curved portion) and CL_{int} (linear portion) of the biphasic plot. It should be noted that the CL_{int} parameter from the biphasic equation pertains to the linear portion of the kinetic plot and not the V_m/K_m ratio for the curved portion (i.e. it represents the slope of the linear portion only). For CYP2C9.3 and CYP2C9.5 in the presence of 0–2 μM dapsone the data were best fit to a linear equation with CL_{int} representing the slope of the line and not the V_m/K_m ratio, since no hyperbolic character was noted within the substrate concentration range studied. The remaining data for each of the variants in the presence of 10–100 μM dapsone (CYP2C9.1) or 5–100 μM dapsone (CYP2C9.2, CYP2C9.3, and CYP2C9.5) were best fit to the Michaelis–Menten equation. Kinetic parameter estimates are represented in Table 5. As with flurbiprofen, dapsone increased the V_m of naproxen demethylation, while the K_m was decreased for all variants. As implied above, the rates of naproxen demethylation became increasingly hyperbolic with increasing dapsone concentration in the case of all CYP2C9 variants. The fold change in efficiency (V_m/K_m) of CYP2C9 mediated naproxen demethylation in the presence of dapsone increased to varying degrees depending on the variant studied (Table 3). For naproxen demethylation, the fold change in efficiency was determined by comparing the V_m/K_m in the presence of 100 μM dapsone with the V_m/K_m in the presence of 5 μM dapsone for CYP2C9.2, CYP2C9.3, and CYP2C9.5, or 10 μM dapsone for CYP2C9.1. Because of the differences in kinetic profiles of naproxen demethylation observed at 0, 2, and 5 μM dapsone as compared to higher dapsone concentrations, it was not possible to make direct comparisons among all the datasets and thus only comparisons of parameters estimated by the Michaelis–Menten equation were conducted. This results in a conservative estimate of the changes.

Table 2
Application of the Michaelis–Menten equation to estimate kinetic parameters for activation of flurbiprofen 4′-hydroxylation by dapsone[a]

Dapsone (μM)	CYP2C9.1			CYP2C9.2			CYP2C9.3			CYP2C9.5		
	V_m (pmol/min/pmol P450)	K_m (μM)	V_m/K_m (μl/min/pmol P450)	V_m (pmol/min/pmol P450)	K_m (μM)	V_m/K_m (μl/min/pmol P450)	V_m (pmol/min/pmol P450)	K_m (μM)	V_m/K_m (μl/min/pmol P450)	V_m (pmol/min/pmol P450)	K_m (μM)	V_m/K_m (μl/min/pmol P450)
0	3.25 (0.28)	33.5 (9.35)	0.10	2.13 (0.06)	29.2 (2.68)	0.07	0.76 (0.03)	170 (15.7)	0.004	1.32 (0.11)	93.1 (21.1)	0.01
2	3.18 (0.25)	29.9 (7.87)	0.11	2.29 (0.06)	23.8 (2.18)	0.10	0.89 (0.05)	133 (15.6)	0.007	1.19 (0.14)	72.5 (24.0)	0.02
5	3.29 (0.32)	25.6 (8.64)	0.13	2.80 (0.09)	15.2 (1.74)	0.18	1.13 (0.03)	96.1 (7.19)	0.012	1.30 (0.20)	63.7 (28.6)	0.02
10	3.52 (0.26)	26.0 (6.46)	0.14	3.42 (0.08)	10.8 (0.94)	0.32	1.46 (0.02)	73.5 (2.72)	0.020	1.30 (0.24)	58.9 (32.5)	0.02
50	4.48 (0.27)	8.48 (2.01)	0.53	5.59 (0.11)	5.0 (0.41)	1.12	3.85 (0.08)	41.4 (2.66)	0.093	2.75 (0.40)	20.3 (10.4)	0.14
100	5.72 (0.28)	7.26 (1.45)	0.79	6.40 (0.20)	2.92 (0.47)	2.19	4.47 (0.13)	23.8 (2.33)	0.188	3.58 (0.20)	16.1 (3.31)	0.22

[a] Data for each concentration of dapsone was fit individually. Parameter estimates are reported as the estimate (standard error of the estimate) resulting from nonlinear regression of the mean of duplicate determinations (<15% variation in all cases).

Table 3
Fold increase in V_m/K_m resulting from dapsone activation of CYP2C9 variant-mediated flurbiprofen 4′-hydroxylation and naproxen demethylation

Variant	Flurbiprofen 4′-hydroxylation	Naproxen demethylation
CYP2C9.1	8-fold	7-fold
CYP2C9.2	31-fold	15-fold
CYP2C9.3	47-fold	13-fold
CYP2C9.5	22-fold	22-fold

Fold increases in V_m/K_m were calculated from the V_m and K_m estimates in Tables 2 and 5 by comparing V_m/K_m in the presence of 100 μM dapsone with V_m/K_m in the absence of dapsone for flurbiprofen 4′-hydroxylation and in the presence of 5 μM dapsone (CYP2C9.2, CYP2C9.3, and CYP2C9.5) or 10 μM dapsone (CYP2C9.1) for naproxen demethylation.

4. Discussion

In addition to allelic differences in substrate metabolism by cytochrome P450 enzymes, other factors can also confound determination of accurate in-vitro/in-vivo correlations, including atypical kinetics, such as positive heterotropic cooperativity or activation. CYP2C9 exhibits all of these characteristics, including polymorphisms and atypical kinetic profiles. We have previously demonstrated that dapsone can activate CYP2C9 mediated metabolism of flurbiprofen and naproxen [11] and that variants of CYP2C9 exhibit lower substrate turnover [27]. However, whether genetic variants of CYP2C9 exhibit differential activation by enzyme effectors such as dapsone has not

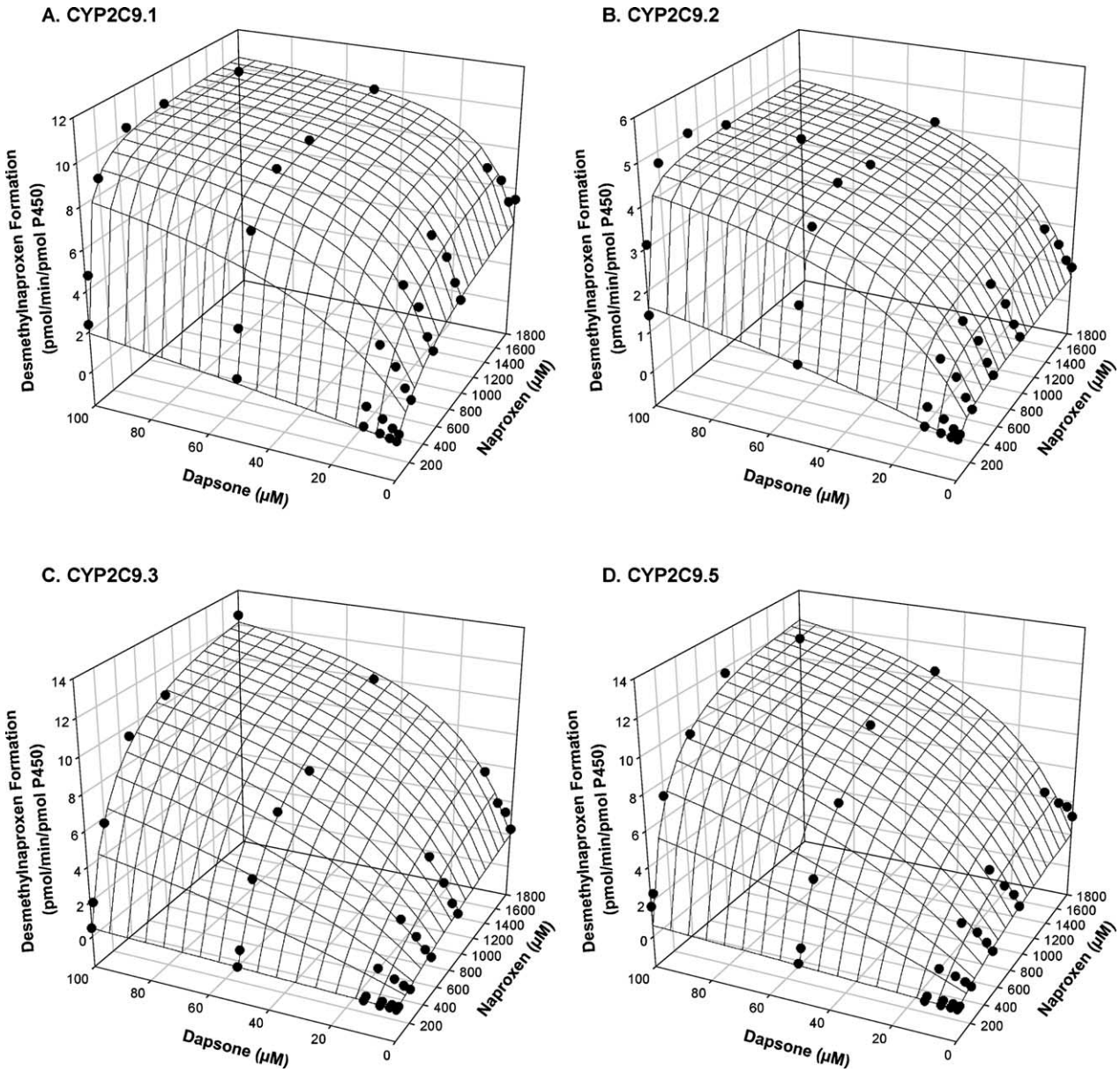

Fig. 2. Dapsone activation of naproxen demethylation in (A) CYP2C9.1, (B) CYP2C9.2, (C) CYP2C9.3, and (D) CYP2C9.5. Scaling of each plot differs to permit better visualization of degree of activation. Data are presented as mean of duplicate determinations, with deviations between determinations <15% in all cases.

Table 4
Kinetic parameter estimates for dapsone activation of naproxen demethylation[a]

Variant	V_m (pmol/min/pmol P450)	K_m (μM)	K_B (μM)	α	β	R^2
CYP2C9.1	4.93 (0.38)	410.4 (81.0)	216.3 (144.0)	0.05 (0.03)	2.34 (0.21)	0.985
CYP2C9.2	0.65 (0.08)	48.4 (13.4)	65.6 (32.9)	0.50 (0.27)	10.50 (1.19)	0.987
CYP2C9.3	2.83 (0.60)	1187.8 (424.4)	120.0 (62.0)	0.16 (0.11)	5.95 (1.54)	0.988
CYP2C9.5	2.53 (0.61)	655.6 (305.5)	160.6 (157.9)	0.17 (0.21)	6.81 (2.05)	0.971

[a] Data fit to two-site model (Eq. (4)). Parameter estimates are reported as the estimate (standard error of the estimate) resulting from nonlinear regression of the mean of duplicate determinations (<15% variation in all cases). K_B, the binding constant for the effector; α, the change in K_m resulting from effector binding; and β, the change in V_m resulting from effector binding. An α value <1 indicates a decrease in K_m. A β value >1 indicates an increase in V_m.

been studied. It was hypothesized that since variants of this enzyme exhibit reduced turnover of substrate, they might also exhibit differential activation of CYP2C9 mediated metabolism by dapsone as compared to wild type enzyme. The effects of dapsone on flurbiprofen and naproxen metabolism by CYP2C9.2, CYP2C9.3, and CYP2C9.5 were studied and compared to results with the wild-type enzyme, CYP2C9.1. These findings demonstrate that genetic variants of CYP2C9 exhibit differential degrees of dapsone activation as measured by differences in the kinetic parameters derived from the two-site model (Tables 1 and 4) as well as by increases in enzyme efficiency (V_m/K_m) for flurbiprofen 4′-hydroxylation and naproxen demethylation (Tables 2 and 5). However, as will be discussed below, it is unclear whether these genetic variations affect substrate and/or effector binding, alter folding of the protein or increase the efficiency of the enzyme through other mechanisms. Recent work in our laboratory has demonstrated that dapsone decreases the uncoupling of the P450 2C9 catalysis of flurbiprofen 4′-hydroxyation by increasing the product/NADPH ratio and reducing the formation of hydrogen peroxide and water, by-products of uncoupling [13]. We have also demonstrated that in the presence of dapsone, the flurbiprofen molecule is moved closer to the heme iron of P450 2C9 resulting in an orientation more amenable to oxidation [10]. Loida and Sligar [20] suggested that the more proximal the site of oxidation to the heme the greater the propensity for a productive reaction to occur. The degree of uncoupling of flurbiprofen metabolism in the variants of CYP2C9 has yet to be determined. However, it is possible that if less productive coupling of the reaction is occurring in the variants as compared to wild type enzyme, dapsone could exert a greater effect in these variants because of a greater "range" of possible improvement in efficiency.

Since changes in metabolism by CYP2C9 allelic variants are substrate dependent, it is necessary to examine dapsone activation with more than one probe substrate. Flurbiprofen hydroxylation was used as a model reaction to monitor dapsone activation of CYP2C9 variants because this reaction has been shown to be activated by dapsone in the wild type enzyme and it exhibits classical Michaelis–Menten kinetics [11]. Our findings demonstrate that CYP2C9 polymorphisms not only affect flurbiprofen 4′-hydroxylation but also the magnitude of activation by effector molecules, such as dapsone. The demethylation of naproxen by variants of CYP2C9 also was tested for differential effects of dapsone activation. Naproxen demethylation by CYP2C9.1 exhibits biphasic kinetics, presumably due to occupation of multiple binding regions within the enzyme active site [11,17]. Additionally, co-incubation with dapsone results in the conversion of the biphasic kinetic profile to a hyperbolic kinetic profile possibly due to the occupation of one of the binding regions by dapsone [11]. Fig. 3 depicts a proposed kinetic scheme for the activation of CYP2C9 mediated naproxen demethylation with multiple substrate/effector binding regions within the active site [17]. Because of this atypical kinetic nature of naproxen demethylation, the role of allelic variants in degree of activation also was studied with this reaction since differences might be expected as compared to compounds exhibiting hyperbolic kinetics (e.g. flurbiprofen). It is theorized that in each of the variants, the presence of dapsone occupies a binding region within the active site leaving one region still available for binding of a single naproxen molecule. It is further proposed that dapsone increases the efficiency of naproxen

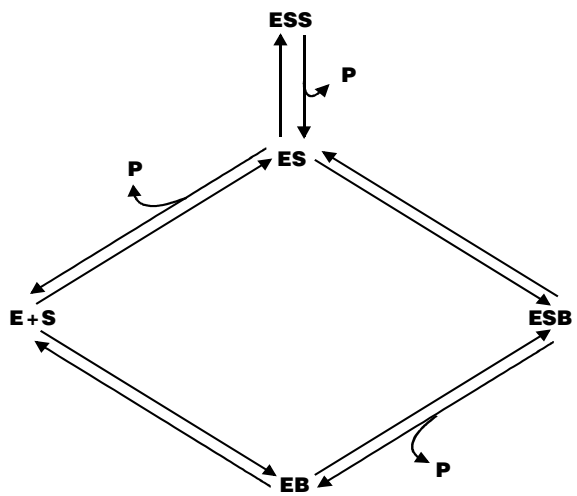

Fig. 3. Simplified kinetic scheme for an enzyme with two substrate/effector binding regions within the active site as proposed to occur with dapsone activation of CYP2C9 mediated naproxen metabolism. Scheme adapted from Korzekwa et al. [17].

Table 5
Application of either a biphasic equation, linear equation or the Michaelis–Menten equation to estimate kinetic parameters for activation of naproxen demethylation by dapsone[a]

Dapsone (μM)	CYP2C9.1			CYP2C9.2			CYP2C9.3			CYP2C9.5		
	V_m (pmol/min/pmol P450)	K_m (μM)	CL_{int} (μl/min/pmol P450)	V_m (pmol/min/pmol P450)	K_m (μM)	CL_{int} (μl/min/pmol P450)	V_m (pmol/min/pmol P450)	K_m (μM)	CL_{int} (μl/min/pmol P450)	V_m (pmol/min/pmol P450)	K_m (μM)	CL_{int} (μl/min/pmol P450)
0	2.58 (0.36)	248 (59.7)	0.0020 (0.0001)	0.70 (0.12)	122 (54.3)	0.0002 (0.0001)	–	–	0.0013 (0.0001)	–	–	0.0017 (0.0000)
2	5.40 (0.97)	408 (120)	0.0006 (0.0004)	1.15 (0.12)	132 (35.5)	0.0000 (0.0001)	–	–	0.0019 (0.0000)	–	–	0.0020 (0.0001)
5	6.75 (0.52)	288 (42.6)	0.0003 (0.0002)	–	–	–	–	–	–	–	–	–
			V_m/K_m (μl/min/pmol P450)			V_m/K_m (μl/min/pmol P450)			V_m/K_m (μl/min/pmol P450)			V_m/K_m (μl/min/pmol P450)
5	–	–	–	1.64 (0.04)	94.3 (11.2)	0.017	8.85 (1.73)	2292 (699)	0.004	7.60 (0.31)	1768 (120)	0.004
10	7.68 (0.16)	218 (16.9)	0.035	2.02 (0.06)	76.6 (10.9)	0.026	11.6 (1.79)	1909 (482)	0.006	7.48 (0.70)	1266 (223)	0.006
50	10.50 (0.17)	72.0 (5.83)	0.146	4.48 (0.12)	32.8 (5.17)	0.137	11.5 (0.65)	357 (61.6)	0.032	13.20 (0.92)	375 (78.5)	0.035
100	10.70 (0.32)	44.2 (7.49)	0.242	4.52 (0.46)	17.7 (11.7)	0.255	14.4 (0.45)	289 (29.7)	0.050	12.70 (0.74)	144 (35.9)	0.088

[a] Parameter estimates are reported as the estimate (standard error of the estimate) resulting from nonlinear regression of the mean of duplicate determinations (<15% variation in all cases). Table is split into biphasic equation (CYP2C9.1 and CYP2C9.2) and linear equation (CYP2C9.3 and CYP2C9.5) fits of the data (top) and Michaelis–Menten fits of the data (bottom).

demethylation at the single site, thus ''normalizing'' the kinetics [11]. This ''normalization'' of kinetic profile has also been observed with CYP3A4 by Nakamura et al. who demonstrated that androstenedione could activate CYP3A4 mediated carbamazepine metabolism and convert the kinetic profile from a sigmoidal to a hyperbolic kinetic form [21].

CYP2C9.2 has been associated with reduced metabolism both in vitro and in vivo, mainly by reducing the V_m, with little effect on K_m. Because of this, amino acid position 144 is not thought to be in the active site of the enzyme. Crespi and Miller have implicated this substitution in reducing the interaction of the P450 with NADPH reductase, which is essential for electron flow necessary for the oxidation of substrates [2]. However, in this study, the presence of dapsone decreased the K_m for both flurbiprofen 4′-hydroxylation and naproxen demethylation mediated by CYP2C9.2, possibly suggesting that alterations in substrate binding in the presence of dapsone may also play a role. The increase in catalytic efficiency and reduction in uncoupling reported during dapsone activation of flurbiprofen 4′-hydroxylation [13] may be due to improvement in electron transfer and may contribute to the substantial increase in substrate turnover observed in the CYP2C9.2 variant.

The CYP2C9.3 variant has a leucine residue in place of an isoleucine residue at amino acid position 359, while the CYP2C9.5 variant contains a glutamate in place of an aspartate at position 360. Both position 359 and 360 have been purported to be in substrate recognition site (SRS) 5 and may play a role in substrate binding [6]. Though these amino acid changes appear minor, the effects on substrate turnover are substantial. Bhasker et al. [1] have suggested that the I359L substitution disturbs the β-strand secondary structure in the substrate recognition site. Since amino acid position 360 is adjacent to 359, it seems plausible that a minor substitution at the 360 position may also cause a slight change in the secondary structure producing differences in substrate metabolism efficiency. More recently, a crystal structure of CYP2C9 has been published [30] and these authors suggested that the amino acid at position 359 is not directly oriented into the active site but may impact active site structure indirectly through interactions with residues within the active site. Whether dapsone perturbs binding of substrate to this portion of the active site and whether dapsone binding is affected by these changes remains to be studied.

Not only were the effects of dapsone activation variant specific, but they were also substrate specific, as has been shown previously with respect to substrate turnover [26,27]. In this study, flurbiprofen 4′-hydroxylation was minimally activated in the presence of 2–10 μM dapsone, while naproxen demethylation was activated to a greater extent at lower dapsone concentrations. Also, the maximal velocity of naproxen demethylation was increased to a level double that of flurbiprofen 4′-hydroxylation in all variants with the exception of CYP2C9.2. By testing a number of steroids with a variety of substrates, Nakamura et al. demonstrated that there are substrate specific differences in effects of endogenous steroids on CYP3A4 mediated metabolism [21]. In addition, Galetin et al. showed that quinidine differentially activated or inhibited CYP3A4 mediated metabolism depending on the substrate probed [5]. Similarly, we have demonstrated that dapsone and analogous compounds activate or inhibit CYP2C9 mediated metabolism to different extents based on the substrate being studied. For example, phenylsulfone activated flurbiprofen metabolism and inhibited naproxen metabolism, *p*-tolyl sulfone activated naproxen metabolism with little effect on flurbiprofen metabolism, and 4-(4-nitrophenylsulfonyl) aniline inhibited flurbiprofen metabolism with little effect on naproxen metabolism [12]. Dapsone appears to exhibit a ''dual binding mode'' and different naproxen metabolism kinetic profiles based on the variant studied, making the finding of substrate differences in degree of activation in the variants not surprising. One can imagine scenarios wherein differences in binding of naproxen molecules in the variants (one molecule binding (linear kinetics) versus two molecules of naproxen binding (biphasic kinetics)) alter dapsone's effects. In these different situations, the ability of dapsone to improve catalytic efficiency through changes in naproxen orientation (as occurs with flurbiprofen) might be differentially affected depending on the variant, substrate binding orientation and the number of naproxen molecules binding.

It is also interesting to note that in all cases, dapsone was able to activate the turnover of substrate to a level similar to or greater than the level achieved by CYP2C9.1 in the absence of dapsone. The presence of dapsone enables the deficient protein to overcome the reduction in substrate turnover caused by the mutations. In the absence of dapsone, CYP2C9.2, CYP2C9.3, and CYP2C9.5 exhibited a V_m/K_m ratio for flurbiprofen metabolism that was 70, 4, and 10%, respectively, that of the wild type enzyme. However, when comparing the V_m/K_m ratio for flurbiprofen 4′-hydroxylation by CYP2C9.2, CYP2C9.3, and CYP2C9.5 in the presence of 100 μM dapsone to CYP2C9.1 in the absence of dapsone, the V_m/K_m ratio is substantially increased, suggesting that the efficiency of the CYP2C9 variants can be altered to equal or exceed that of the wild type enzyme. For naproxen demethylation, it is more difficult to compare the kinetic parameters due to the different kinetic profiles for the absence and presence of dapsone. However, from Fig. 3, it is clear that naproxen demethylation is increased to a greater level for all of the variants studied as compared to wild-type CYP2C9, especially at lower concentrations of naproxen. Whether this ability to convert a ''poor'' metabolizer to an extensive metabolizer state in vitro can be translated to the in vivo situation remains to be studied. With respect to drugs exhibiting a more narrow therapeutic range than the NSAIDs, a recent abstract report has suggested that lansoprazole can activate in vitro metabolism of phenytoin by

400% [19], suggesting the possibility of regulating the metabolism of a therapeutically critical drug (phenytoin). Taken together, these findings raise intriguing possibilities in terms of the potential ability to ''normalize'' patients exhibiting CYP2C9 polymorphisms with respect to drug metabolism capacity.

In summary, the ability of dapsone to activate CYP2C9 mediated metabolism is both variant and substrate dependent. Dapsone co-incubation reduces the K_m and increases the V_m of both flurbiprofen 4′-hydroxylation and naproxen demethylation resulting in an increase in the overall efficiency of the enzyme with greater effects being noted in the variants as compared to wild type enzyme. These findings demonstrate that the degree of drug–drug interaction may be influenced by an individual's genotype.

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