Diversity in the Oxidation of Substrates by Cytochrome P450 2D6: Lack of an Obligatory Role of Aspartate 301−Substrate Electrostatic Bonding†

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ABSTRACT: Cytochrome P450 (P450) 2D6 was first identified as the polymorphic human debrisoquine hydroxylase and subsequently shown to catalyze the oxidation of a variety of drugs containing a basic nitrogen. Residue Asp301 has been characterized as being involved in electrostatic interactions with substrates on the basis of homology modeling and site-directed mutagenesis experiments [Ellis, S. W., Hayhurst, G. P., Smith, G., Lightfoot, T., Wong, M. M. S., Simula, A. P., Ackland, M. J., Sternberg, M. J. E., Lennard, M. S., Tucker, G. T., and Wolf, C. R. (1995) *J. Biol. Chem. 270*, 29055−29058]. However, pharmacophore models based on the role of Asp301 in substrate binding are compromised by reports of catalytic activity toward substrates devoid of a basic nitrogen, which have generally been ignored. We characterized a high-affinity ligand for P450 2D6, also devoid of a basic nitrogen atom, spirosulfonamide [4-[3-(4-fluorophenyl)-2-oxo-1-oxaspiro[4.4]non-3-en-4-yl]benzenesulfonamide], with K_s 1.6 μM. Spirosulfonamide is a substrate for P450 2D6 (k_{cat} 6.5 min^{-1} for the formation of a *syn* spiromethylene carbinol, K_m 7 μM). Mutation of Asp301 to neutral residues (Asn, Ser, Gly) did not substantially affect the binding of spirosulfonamide (K_s 2.5−3.5 μM). However, the hydroxylation of spirosulfonamide was attenuated in these mutants to the same extent (90%) as for the classic nitrogenous substrate bufuralol, and the effect of the D301N substitution was manifested on k_{cat} but not K_m. Analogues of spirosulfonamide were also evaluated as ligands and substrates. Analogues in which the sulfonamide moiety was modified to an amide, thioamide, methyl sulfone, or hydrogen were ligands with K_s values of 1.7−32 μM. All were substrates, and the methyl sulfone analogue was oxidized to the *syn* spiromethylene carbinol analogue of the major spirosulfonamide product. The D301N mutation produced varying changes in the oxidation patterns of the spirosulfonamide analogues. The peptidometic ritonavir and the steroids progesterone and testosterone had been reported to be substrates for P450 2D6, but the affinities (K_s) were unknown; these were estimated to be 1.2, 1.5, and 15 μM, respectively (cf. 6 μM for the classic substrate bufuralol). The results are consistent with a role of Asp301 other than electrostatic interaction with a positively charged ligand. H-Bonding or electrostatic interactions probably enhance binding of some substrates, but our results show that it is not required for all substrates and explain why predictive models fail to recognize the proclivity for many substrates, especially those containing no basic nitrogen.

P450s[1] are major enzymes involved in the oxidations of many organic chemicals (*2*, *3*). Particular interest has been given to the mammalian P450 enzymes that dominate the metabolism of drugs (*4*). Variability among individuals can have a major influence on the efficacy of drugs, and human P450s are an important target of efforts in pharmacogenomics (*5*).

P450 2D6 was first identified as the polymorphic debrisoquine hydroxylase (*6*, *7*). This enzyme is estimated to be involved in the metabolism of ∼30% of the drugs on the market today, more than any other P450 except P450 3A4 (*4*). Today the polymorphism of P450 2D6 is relatively well understood, at least in terms of the polymorphisms that exist (*6*−*8*), and efforts have been directed to better understand the biochemical basis of P450 2D6 activity. In early research

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[1] Abbreviations: P450, microsomal cytochrome P450 [also termed "heme-thiolate protein P450" (*1*)]; 1D, one dimensional; 2D, two dimensional; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; MS, mass spectrometry; HO, hydroxy; NOE, nuclear Overhauser effect; DFQ, double-filtered quantum; COSY, correlated spectroscopy; HMQC, heteronuclear multiple quantum correlation.

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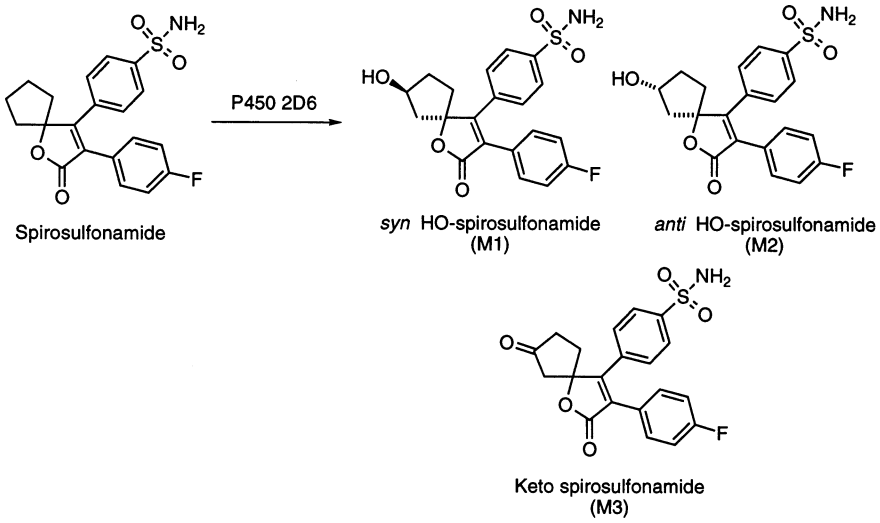
Scheme 1: Spirosulfonamide and Oxidation Products



with purified P450 2D6 in this laboratory (*9*), the observation was made that most of the known substrates for P450 2D6 contained a basic nitrogen atom, which is located 5−7 Å away from the site of oxidation on the substrate (*10*, *11*). This concept was developed with more detailed models of P450 2D6, in terms of both pharmacophore models and homology modeling and combinations of the two (*12*−*14*). Site-directed mutagenesis of Asp301 led to loss of catalytic activity in yeast recombinant P450 2D6 (*15*), and the result has been widely interpreted as evidence that this acidic amino acid serves as a point anionic charge to dock the basic nitrogen atom of the inhibitor (*11*) or substrate (*16*−*20*). For example, one often finds statements such as the following in the current literature on P450 2D6: "All of our data [on propranolol derivatives] are consistent with the generally accepted model for binding of CYP2D6 [P450 2D6] substrates via formation of an ion pair of the protonated amine with the carboxylate anion of Asp301 in the enzyme active site and subsequent oxidation at a distant site in the molecule" and "The presence of basic nitrogens is a common requirement of high-affinity substrates..." (*21*).

One problem with models of the P450 2D6 structure (and pharmacophore models) was that they did not rationalize N-dealkylation reactions, such as those observed with deprenyl (*22*) and other drugs (*23*). One explanation for this dilemma was that the amine docks with Asp301 but is rapidly deprotonated to yield an unchanged substrate for 1-electron oxidation (*22*). Subsequently, models were expanded to accommodate this deficiency, with aromatic residues involved (*24*, *25*). Experimental studies have provided only limited and rather nongeneralized support for roles of these suggested Phe residues (Phe481, Phe483) (*25*, *26*). Further, reports have appeared of catalytic activity of P450 2D6 toward peptidometics devoid of a basic nitrogen (*27*) and even steroids (*28*−*30*). However, these reports have been largely ignored in consideration of P450 2D6 substrates (*21*).

We identified a ligand, spirosulfonamide, devoid of a basic nitrogen but having high affinity for P450 2D6, raising further concerns about the reliability of P450 2D6 models based on a critical electrostatic interaction with Asp301. Spirosulfonamide was also a substrate for P450 2D6 (Scheme 1). Neutral Asp301 mutants (Asn, Ser, Gly) showed relatively high affinity for spirosulfonamide (K_d 10^{-6} M). The compromised catalytic activity of these mutants for spirosulfonamide hydroxylation parallels that observed with the basic substrate bufuralol, and attenuated electrostatic interaction of substrate cannot be used as an explanation of the role of Asp301. P450 2D6 also bound and oxidized analogues of spirosulfonamide in which the sulfonamide group was replaced by other groups. We also estimated the affinity of P450 2D6 for other substrates (ritonavir, progesterone, and testosterone) and report binding affinities as good or better than for many classic ligands with basic nitrogen.

EXPERIMENTAL PROCEDURES

Chemicals. Spirosulfonamide (**1**), *syn* and *anti* HO-spirosulfonamides, and keto spirosulfonamide were synthesized according to the procedures outlined in the Supporting Information (*31*). Other spirosulfonamide analogues, compounds **2**−**6**, were experimental drugs that were prepared at Merck-Frosst using synthetic procedures analogous to that used for spirosulfonamide. Ritonavir was a gift of Abbott Laboratories (North Chicago, IL). Bufuralol hydrochloride was a gift of Hoffman-LaRoche (Nutley, NJ). Deuterated NMR solvents were obtained from C/D/N Isotopes (Montreal, Quebec, Canada) and Cambridge Isotope Laboratories (Andover, MA). Other chemicals were obtained from commercial sources and were of reagent, analytical, or HPLC grade, as appropriate.

Microsomal Fractions and Enzymes. Human liver samples were obtained from organ donors through Tennessee Donor Services (Nashville, TN), stored at −80 °C, and used to prepare microsomes (*33*). In some of the preliminary experiments with spirosulfonamide, microsomes prepared from insect cells infected with baculovirus vectors were used as sources of human P450s, NADPH−P450 reductase, and, in the case of P450 3A4, cytochrome b_5 [Gentest Co., Woburn, MA (now BD Bioscience)]. The baculovirus microsome products used were those designated control, CYP2C8, CYP2C9-arg, CYP2C19, CYP2D6, and CYP3A4 by the supplier.

The cDNA sequence of P450 2D6 (DB6) (*34*) was modified by polymerase chain reaction to incorporate a

C-terminal $(His)_5$ peptide (*35*).[2] The modified protein was expressed in *Escherichia coli* (MV1304 strain) in the presence of 1.0 mg of chloramphenicol L^{-1} (*38*). Expressed P450s were purified by Ni^{2+}-immobilized metal affinity chromatography as described (*35*, *39*). Rat NADPH−P450 reductase was expressed in *E. coli* (TOPP 3 strain) utilizing plasmid pOR263 (*40*) and was purified as described (*39*, *41*).

*Biotransformations of Spirosulfonamide (**1**).* Incubations and metabolite characterization by mass spectrometry and NMR are completely detailed in the Supporting Information. Briefly, hepatocyte and preparative microsomal incubations were conducted under standard conditions (*42*). Initial mass spectral characterization was conducted by capillary HPLC continuous-flow liquid secondary ion MS analysis on a JEOL HX-110A mass spectrometer (*43*). Isolation of the major metabolite (M1, subsequently identified as *syn* HO-spirosulfonamide) was performed using a combination of preparative HPLC and solid-phase extraction procedures similar to those described previously (*43*). Conventional ^1H NMR spectra for the isolated metabolite were acquired on a Bruker AMX 500 spectrometer (Bruker Spectrospin, Fremont, CA) using a combination of homonuclear coupling, 1D NOE, and 2D DFQ-COSY and HMQC experiments (*44*).

*Biotransformations of Spiromethyl Sulfone (**2**).* Preparative scale incubations and HPLC and mass spectrometry of the product were done as in the case of **1** (see Supporting Information). HPLC−NMR spectra of the methyl sulfone product were acquired on a Varian Inova 600 spectrometer (Varian NMR Systems, Palo Alto, CA) equipped with a Varian HPLC system (Varian Chromatography Systems, Walnut Creek, CA) using a COSY experiment and by comparison to the parent compound. Full details can be found in the Supporting Information.

Determination of P450s Involved in Spirosulfonamide Oxidation. Preliminary studies involved incubations with microsomes of baculovirus-infected insect cells expressing specific P450s. The incubations were conducted under standard oxidative conditions using 50 pmol of P450 (as stated by the vendor), an NADPH-generating system, and 20 μM spirosulfonamide (dissolved in DMSO, to 1% final volume) in a final total volume of 250 μL. The incubations were conducted for 60 min at 37 °C and quenched by the addition of an equal volume of CH_3CN. Analysis was conducted by HPLC under conditions similar to those described below.

Binding of Spirosulfonamide and Analogues to P450 2D6. Heme perturbation spectra were recorded with purified P450 2D6 enzyme preparations (2−5 μM, in 0.10 M potassium phosphate buffer, pH 7.4) using stocks of spirosulfonamide dissolved in CH_3CN (*45*, *46*). Absorbance spectra were recorded on Aminco DW2a/OLIS and Cary 14/OLIS instruments (On-Line Instrument Systems, Bogart, GA). Other work has indicated that neither DMSO nor CH_3CN hinder P450 2D6 reactions when added at concentrations up to 1.0% (v/v) (*47*). The analogues were also dissolved in CH_3CN and added to P450 2D6 to generate type I perturbation spectra. Values of $A_{390}-A_{420}$ were plotted vs the ligand concentration to estimated K_s, a spectrally estimated dissociation constant (*45*), using Graphpad Prism software (Graphpad, San Diego, CA). Hyperbolic fitting was used with high K_s values, and a quadratic expression was used when K_s was near the P450 concentration or the program DynaFit was applied (see Supporting Information) (*48*).

Some of the compounds tested as ligands did not elicit perturbation spectra, and an alternate approach was used. Bufuralol elicits type I binding spectra ($K_s = 6.2 \pm \mu$M). Two P450 samples were balanced against each other as before in the Cary 14/OLIS spectrophotometer, and then (±)-bufuralol was added to the sample cuvette to 8 μM, a value near the K_s, to induce a type I difference spectrum. Subsequently, varying amounts of CH_3CN solutions of ritonavir, progesterone, or testosterone were added to the cuvette to attenuate the difference spectrum. The competition between the "second" ligand and bufuralol was assumed to be competitive, and plots of $A_{390}-A_{420}$ vs the concentration of the second ligand were fit to the model

$$\text{bufuralol} + \text{P450 2D6} \rightleftarrows \text{bufuralol·P450 2D6} \qquad K_s$$

$$\text{second ligand} + \text{P450 2D6} \rightleftarrows \text{second ligand·P450 2D6} \qquad K_d$$

using the software program DynaFit (*48*) operating on an Apple Macintosh G4 computer, with $K_s = 6.2$ μM (determined experimentally). The system yielded fits for K_d and for $\Delta A_{390-420,max}$, along with error estimates.

Oxidation of Spirosulfonamide and Analogues by P450 2D6. Standard oxidation reactions were conducted in 1.0 mL final volumes of 0.10 M potassium phosphate buffer (pH 7.4) containing either 0.05 or 0.10 nmol of P450 2D6, a 3-fold molar excess of NADPH−P450 reductase, and freshly sonicated L-α-dilauroyl-*sn*-glycero-3-phosphocholine (Sigma Chemical Co., St. Louis, MO) (30 μg). [In cases where human liver microsomes were used instead of purified P450 2D6, 0.75 mg of microsomal protein was substituted for the above components, in the same buffer, in the absence or presence of 2.0 μM quinidine (Aldrich Chemical Co., Milwaukee, WI), in triplicate.] Spirosulfonamide and its analogues were dissolved in CH_3CN to prepare stock solutions [final concentration ≤1% (v/v) in the reactions]. The mixtures were incubated for 3 min at 37 °C, and the reactions were started by the addition of an NADPH-generating system (*33*). Incubations were carried out for 5 min at 37 °C.

Reactions were stopped by the addition of 2.0 mL of CH_2Cl_2 and chilled on ice, followed by mixing using a vortex device. The layers were separated by centrifugation [$(3 \times 10^4)g$, 10 min]. A 1.6 mL aliquot of each CH_2Cl_2 (lower) layer was removed with a glass pipet, transferred to a 2.0 mL Reacti-vial (Pierce Chemical Co., Rockford, IL), and concentrated to dryness under an N_2 stream. The residues were dissolved in 100 μL of CH_3CN, and 50 μL aliquots were analyzed by HPLC using a 6.2 × 80 mm Zorbax octylsilane (C_8) HPLC column (3 μm; Mac-Mod, Chadds Ford, PA). Solvent A was 1.0 mM $HClO_4$ and solvent B was CH_3CN-H_2O (9:1 v/v). The elution program consisted of a linear gradient increasing from 5% to 75% solvent B (v/v) over the following 20 min (flow rate 2.5 mL min^{-1},

[2] The cDNA we expressed in previous work (*34*) had been obtained with a change (to Met) at codon 374 that appears to be the result of a cloning artifact (*36*, *37*). The change M374V was made, and the resulting sequence is that generally agreed to be the most common allele (*6*).

A_{245}). The alcohols (*syn* and *anti* HO-spirosulfonamide) and ketone (keto spirosulfonamide) were separated with baseline resolution (vide infra).

RESULTS

Characterization of P450-Catalyzed Oxidations of Spirosulfonamide in Liver Microsomes and Baculovirus Recombinant P450 2D6. Preliminary studies indicated that spirosulfonamide, devoid of a basic nitrogen atom, might be preferentially oxidized by P450 2D6. When spirosulfonamide was incubated with baculovirus-generated microsomes containing individual human P450s (50 pmol of P450, 20 μM spirosulfonamide, 37 °C, 60 min), the decreases in residual spirosulfonamide with each P450 were as follows: 2D6, 84%; 3A4, 43%; 2C8, 20%; 2C9, <5%; 2C19, <5%. Twelve different human liver microsomal samples were incubated with 50 μM spirosulfonamide for 10 min at 37 °C. A product denoted M1 (order of HPLC elution) was the major product, with a second peak denoted M2 accounting for ~15% of the M1 peak in most samples and traces of a third peak, M3. With the preliminary assumption that the ϵ_{245} of M1 is equivalent to that of spirosulfonamide (later verified), the range of specific activity was from <0.01 to 2.0 nmol of M1 formed min^{-1} (mg of protein)$^{-1}$ (mean 0.32). Quinidine is a well-established inhibitor of P450 2D6, and a 2 μM concentration was used to specifically block P450 2D6 (*49*, *50*). The extent of inhibition ranged from <5% to 59%, thus establishing a role for P450 2D6 in liver samples.

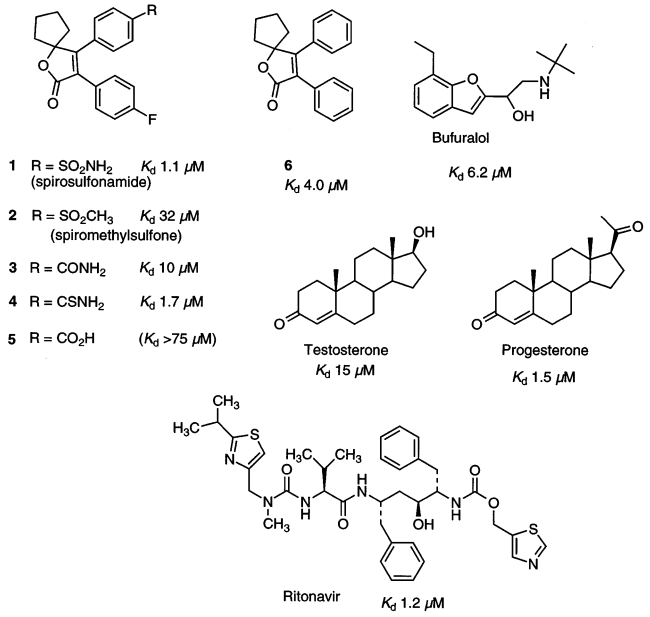
*Characterization of Oxidation Products of Spirosulfonamide (**1**) and Spiromethyl Sulfone (**2**).* HPLC−MS analysis of spirosulfonamide incubates and NMR chararcterization of isolated M1 generated from spirosulfonamide (**1**) allowed complete characterization of the three metabolites (see Supporting Information). M1 and M2 were monohydroxylated *syn* and *anti* HO-spirosulfonamides (M + 16 products by MS), and M3 (corresponding to the net addition of 14 Da) was the corresponding ketone. Furthermore, incubation of both the *syn* and *anti* HO-spirosulfonamides in rat hepatocytes produced the ketone (results not shown). The two M + 16 products each showed a clear $MH^+ - H_2O$ peak in the spectra, indicative of dehydration and suggesting hydroxylation of the cyclopentyl structure. NMR data supported this conclusion and indicated hydroxylation had occurred on the "outer" methylene of the cyclopentyl moiety. Full details can be found in the Supporting Information.

The structure of the oxidation product of the spiromethyl sulfone analogue **2** (Scheme 2) was determined using HPLC−NMR methodology, and the major product was determined to be analogous to that of the spirosulfonamide metabolite, M1 (see Supporting Information).

Binding of Spirosulfonamide and Analogues to Purified P450 2D6. The interaction of many, but not all, substrates with P450s is characterized by a "type I" difference spectrum (*45*), reflecting decreased occupancy of the axial iron binding site by H_2O and a shift from the low-spin to the high-spin iron configuration. Spirosulfonamide yielded strong, classic type I heme perturbation spectra with recombinant P450 2D6 (Figure 1A), and a titration yielded a K_s of 1.6 μM (Figure 1B).

A number of analogues of spirosulfonamide were available with substitutions of the sulfonamide group (Scheme 2).

Scheme 2: Analogues of Spirosulfonamide and Other P450 2D6 Ligands[a]



[a] K_d values were estimated by spectral titrations (Table 1).

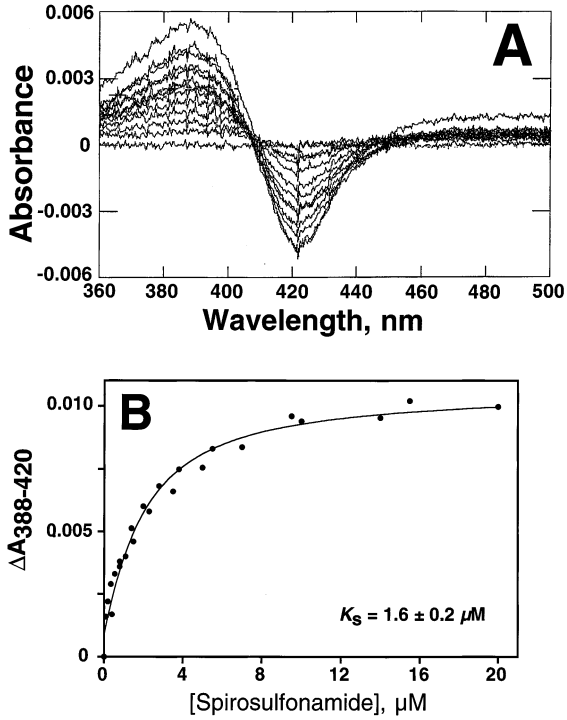


FIGURE 1: Titration of P450 2D6 with spirosulfonamide. (A) P450 2D6 (wild type) was present in each of two cuvettes (1.0 μM, in 0.10 M potassium phosphate buffer). Difference spectra were recorded following the additions of 0, 0.4, 0.8, 1.4, 2.0, 2.8, 3.8, 5.0, 7.0, 10, 14, and 20 μM spirosulfonamide (dissolved in CH_3CN, with CH_3CN added to the reference at each point; total [CH_3CN] = 2.5%, v/v). (B) Plot of data from part A. The estimated K_s was 1.6 μM.

These compounds were examined as ligands with (ferric) P450 2D6. All except **5** yielded type I difference spectra, with varying affinities (Table 1). Compounds with an $-SO_2NH_2$ and $-CSNH_2$ had the highest affinities (K_s < 2 μM), but compound **6**, with no phenyl substitution, also yielded K_s = 4 μM.

Table 1: Spectrally Determined Binding of Other Potential Ligands to P450 2D6

compound	functional group[a]	type of spectral change	K_d[b],[c] (μM)	$\Delta A_{390-420,max}$[c] [(μM P450)$^{-1}$]
spirosulfonamide (**1**)	$-SO_2NH_2$	type I	1.1 ± 0.02	$(10.5 \pm 0.3) \times 10^{-3}$
spiromethyl sulfone (**2**)	$-SO_2CH_3$	type I	32 ± 1	$(9.3 \pm 0.1) \times 10^{-3}$
3	$-CONH_2$	type I	10 ± 1	$(14.0 \pm 0.5) \times 10^{-3}$
4	$-CSNH_2$	type I	1.7 ± 0.3	$(5.2 \pm 0.2) \times 10^{-3}$
5	$-CO_2H$	none		
6	$-H$[d]	type I	4.0 ± 0.5	$(8.3 \pm 0.2) \times 10^{-3}$
testosterone		none[e]	15 ± 4	
progesterone		none[e]	1.5 ± 0.3	
ritonavir		none[e]	1.2 ± 0.6	
(±)-bufuralol		type I	6.2 ± 1.8	$(24 \pm 4) \times 10^{-3}$

[a] At the para position of the phenyl ring of spirosulfonamide, when indicated (not relevant to steroids, ritonavir, or bufuralol). [b] K_d presented as K_s for type I changes ($\lambda_{max} \sim 390$ nm, $\lambda_{min} \sim 420$ nm in difference spectrum). Established by competition with bufuralol (present at 7 or 8 μM). [c] ± indicates SE. [d] No fluorine in this analogue (Scheme 2). [e] No spectral change. Competes with bufuralol. See Figure 2. The estimates were made using the DynaFit script described under Experimental Procedures (see Supporting Information for full script).

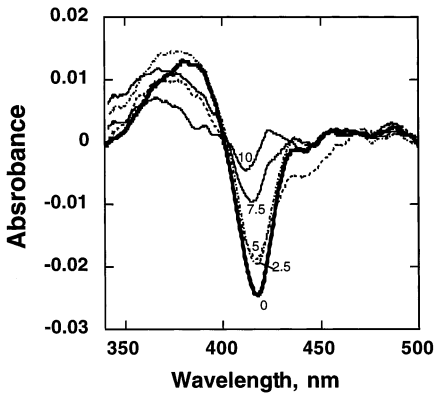


FIGURE 2: Displacement of bufuralol from P450 2D6 by ritonavir. Cuvettes containing 5.0 μM P450 2D6 (with 45 μM di-12:0 GPC and 0.10 M potassium phosphate buffer, pH 7.4) were balanced against each other, and then 8 μM (±) bufuralol hydrochloride ($\sim K_s$) was added to the sample cuvette to yield the spectrum designated 0. The spectra labeled 2.5, 5, 7.5, and 10 were recorded after the addition of the indicated (μM) concentration of ritonavir to the sample cuvette (stock ritonavir in CH_3CN, with equal volumes of CH_3CN added to the reference cuvette; total $CH_3CN < 1\%$, v/v).

P450 2D6 has been reported to catalyze oxidations of ritonavir and progesterone, with K_m values of 10 and 29 μM, respectively (respective k_{cat} values of 1.4 and $\sim$0.5 min^{-1}) (*27*, *29*). A rate of $\sim$2 min^{-1} has been reported for P450 2D6-catalyzed testosterone hydroxylation (sum of reactions at several sites), but no K_m was determined (*28*). A K_i of 68 μM has been estimated for competitive inhibition of bufuralol 1′-hydroxylation activity (*29*). However, affinities are not known. In the present work, none of these compounds yielded difference spectra. However, affinities could be estimated by quantifying their abilities to displace the ligand bufuralol (K_s 6.2 μM) (Figure 2). Using the assumption of competitive binding for a single site, the results were fit to a model using the program DynaFit, yielding $K_d = 15$ μM for testosterone and K_d values less than P450 2D6 concentration for progesterone and ritonavir (and thus having more error) (Table 1).

Effect of Mutation of Asp301 on Binding of Amines. Mutation of Asp301 to neutral residues has already been reported to lower the affinity of P450 2D6 for the amine ligand quinidine (an inhibitor and not a substrate) (*15*). We also found that the mutant D301N yielded only a spectral shift with quinidine (Figure 3). Further studies indicated that the D301N mutant also failed to bind bufuralol, as judged by the lack of type I spectra (results not shown).

Interaction of P450 2D6 Asp301 Mutants with Spirosulfonamide. Several neutral Asp301 mutants had been prepared previously and demonstrated to have low catalytic activity in the oxidation of bufuralol (*35*) and other basic nitrogenous substrates (*15*). In the latter case the attenuation of activity had been proposed to be due to the disruption of an electrostatic bond between Asp301 and the ligand (*15*).

Spectral titrations of the P450 2D6 Asp301 mutants were done with spirosulfonamide. All mutants examined showed typical, strong interaction with spirosulfonamide (Table 2). Thus, mutation of Asp301 to a neutral residue does not attenuate the binding of spirosulfonamide considerably, as reflected by the type I difference spectra.

The oxidation of spirosulfonamide by the Asp301 mutants was also examined (Figure 4, Table 3). All mutants showed attenuated hydroxylation activity (formation of *syn* HO-spirosulfonamide). Two of the mutants formed approximately equal amounts of the two alcohol isomers. The fraction of *anti* HO-spirosulfonamide formed by wild-type P450 2D6 is less than in most human microsomal samples ($\sim$5%), consistent with the results obtained using recombinant P450s, which demonstrated that P450 3A4 also contributes to this reaction (results not shown).

Oxidation of Spirosulfonamide by P450 2D6 and Asp301 Mutants. The oxidation of the spirosulfonamide was examined with several Asp301 mutants at a substrate concentration of 50 μM, including the Asn, Ser, and Gly mutants (Figure 4). The rates of oxidation were decreased about 10-fold, as in the case of bufuralol (*35*), although the rates of formation of *anti* HO-spirosulfonamide were increased in the D301G and D301S mutants (Table 3).

A k_{cat} of 6.5 min^{-1} and K_m of 7.2 μM were determined for the formation of *syn* HO-spirosulfonamide (Figure 5). Incubation of the P450 2D6 system with either (30 μM) *syn* HO-spirosulfonamide or *anti* HO-spirosulfonamide yielded keto spirosulfonamide (respective rates of 3.0 and 0.75 min^{-1}), with two additional unidentified products in the case of *syn* HO-spirosulfonamide (results not presented).

Further analysis of the oxidation of spirosulfonamide indicated that the effect of mutation of Asp301 to Asn was to reduce the k_{cat} (for oxidation of spirosulfonamide) 10-fold without a change of the K_m (Figure 5).

Oxidation of Spirosulfonamide Analogues by Wild-Type P450 2D6 and the D301N Mutant. Preliminary studies with commercial baculovirus-expressed P450 2D6 had shown that all of the compounds yielded some oxidation products, with t_R shorter than that of the substrate on reversed-phase HPLC. The same peaks were also obtained with human liver microsomal samples (results not shown). The oxidation of the spirosulfonamide analogues was examined with wild-type P450 2D6 and the mutant D301N at single substrate concentrations of 50 μM, using the same HPLC system for all analyses.

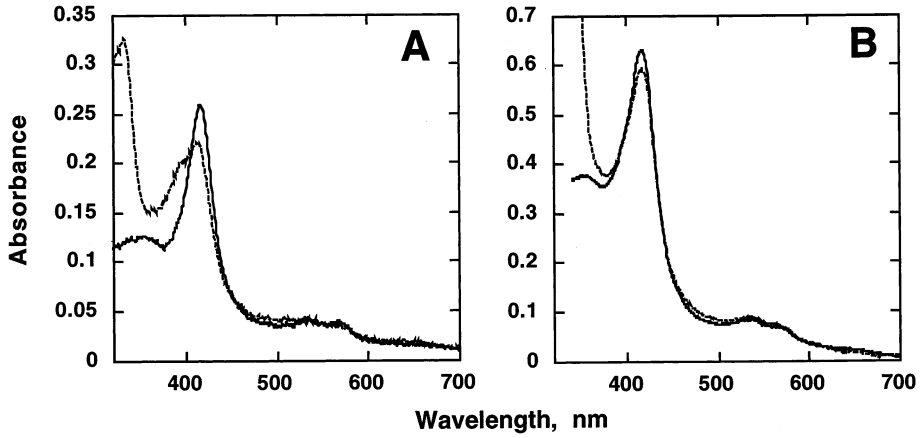


FIGURE 3: Spectra of P450 2D6 without and with added quinidine: minus quinidine, solid line (—); plus quinidine, dashed line (- - -). (A) Wild-type P450 2D6 (2.0 μM) ± 20 μM quinidine. (B) P450 2D6 D301N mutant (4.0 μM) ± 50 μM quinidine. No further change in the spectrum was observed upon the addition of 200 μM quinidine.

Table 2: Spectrally Determined Binding of Spirosulfonamide to Ferric P450 2D6 and Asp301 Mutants

P450 2D6	K_s[a] (μM)	$\Delta A_{390-420,max}$[a] [(μM P450)$^{-1}$]
wild type	1.6 ± 0.2	(10.7 ± 0.3) × 10^{-3}
D301N	2.5 ± 0.3	(9.8 ± 0.3) × 10^{-3}
D301G	3.5 ± 0.5	(9.7 ± 0.3) × 10^{-3}
D301S	3.5 ± 0.7	(11.5 ± 0.5) × 10^{-3}

[a] SE indicated.

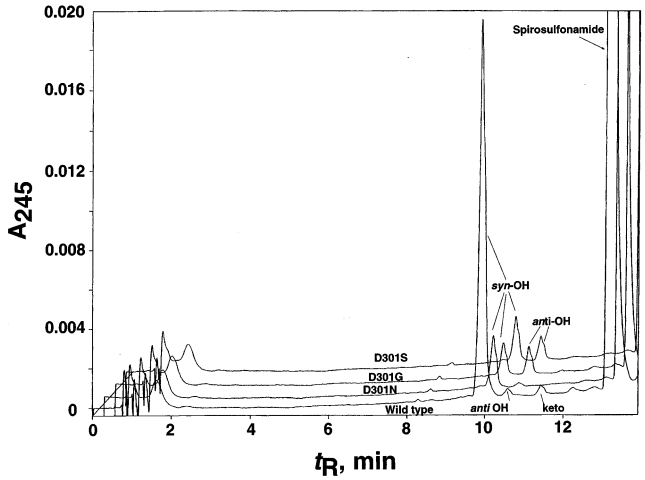
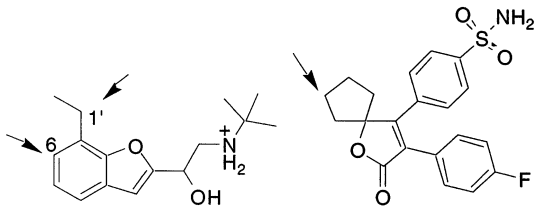


FIGURE 4: Oxidation of spirosulfonamide by wild-type P450 2D6 and Asp301 mutants. HPLC of products of the incubation of 0.10 nmol of P450 2D6 with 30 μM spirosulfonamide for 5 min at 37 °C. The traces are offset 0.3 min in the software system (PC1000/UV3000HR; Thermo-Separations, Piscataway, NJ), and the chromatograms obtained with wild-type P450 2D6 and the D301N, D301G, and D301S mutants are indicated.

As in the case of the baculovirus system, little oxidation of the carboxyl analogue **5** was seen [rate < 0.05 nmol of product formed min^{-1} (nmol of P450)$^{-1}$)]; the other products were formed at rates of <0.5 min^{-1}, using the assumption that the ϵ_{245} of all products is similar to *syn*-OH spirosulfonamide. The effect of substitution of Asp301 to Asn had variable effects. Compound **2**, the methyl sulfone, was oxidized to the same *syn*-alcohol as spirosulfonamide (**1**), with regard to the specific methylene carbon (see Supporting Information). The estimated rates were 0.36 and 0.33 nmol of product formed min^{-1} (nmol of P450)$^{-1}$ for wild-type P450 2D6 and the D301N mutant, respectively.

Table 3: Catalytic Activities [nmol min^{-1} (nmol of P450)$^{-1}$] of P450 2D6 and Asp301 Mutants



	bufuralol[a]		spirosulfonamide[b]	
P450 2D6	1′-OH	6′-OH	*syn* OH	*anti* OH
wild type	21 ± 1	5.0 ± 0.3	7.2 ± 0.4	0.1
D301N	2.4 ± 0.7	0.7 ± 0.1	0.8 ± 0.2	<0.05
D301G	1.9 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
D301S	1.9 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1

[a] From ref *30*. [b] Results are presented as the means of duplicate assays ± SD (range). The substrate concentration was 30 μM in all cases (0.10 nmol of P450, 5 min reaction at 37 °C).

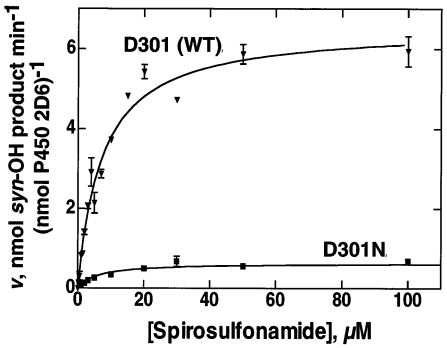


FIGURE 5: Plot of rates of formation of *syn* HO-spirosulfonamide vs concentration of the substrate spirosulfonamide. (A) Wild-type P450 2D6. A fit to the hyperbola yielded estimates of k_{cat} = 6.5 ± 0.2 min^{-1} and K_m = 7.2 ± 0.7 μM (SE indicated). (B) P450 2D6 D301N mutant. Hyperbolic fitting yielded k_{cat} = 0.64 ± 0.03 min^{-1} and K_m = 5.8 ± 1.3 μM (SE indicated).

Oxidation of **3** by wild-type P450 2D6 yielded three products (Figure 6A,B), still unidentified. Substitution of Asn at position 301 attenuated the formation of two of these but enhanced formation of the third (t_R 14.3 min). In the oxidation of compound **6**, devoid of phenyl substitution, the

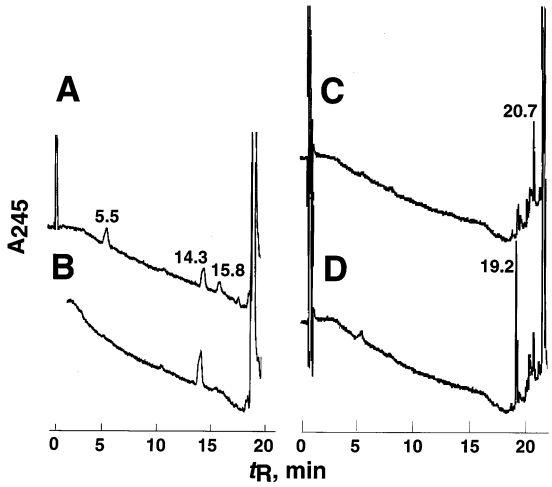


FIGURE 6: HPLC profiles of oxidation of compounds **3** and **6** by wild-type P450 301 and the D301N mutant. In all cases the concentrations of P450 2D6 (D301 or D301N), NADPH−P450 reductase, and the substrate were 0.10, 0.25, and 50 μM, respectively, and the incubation time was 10 min (37 °C). HPLC conditions were as described for spirosulfonamide and its products (e.g., Experimental Procedures and Figure 4). The chromatograms resulted from the incubation of **3** with P450 2D6, either wild type (A) or the D301N mutant (B), or the incubation of **6** with P450 2D6, wild type (C) or the D301N mutant (D). The t_R values (in min) are indicated for the major products. For reference, the estimated rates of formation of the major product in parts A and C are 0.25 and 0.12 nmol min^{-1} (nmol of P450)$^{-1}$, respectively.

HPLC pattern shifted from one of the products to another (Figure 6C,D). In work with compound **4**, mutation of Asp301 to Asn also changed the pattern from a mixture of two products (electing at t_R 10.6 and 15.7 min, ratio 1:2) to a single t_R 10.6 min product, produced at 5 times the rate measured with the wild-type enzyme.

DISCUSSION

P450 2D6 has been of historical interest in that this is the first monoxygenase involved in drug metabolism that was demonstrated to be under monogenic control (*51*). Another reason for biochemical interest in this particular P450 has been the apparent ligand selectivity relative to many less-specific microsomal P450s (*10*, *15*). The ability to produce P450 2D6 and site-directed mutants in heterologous expression systems has allowed exploration of several issues, including the role of the N-terminus (*34*, *35*) and the effects of amino acid substitution at Asp301 (*15*, *35*).

The history of model building for P450 2D6 began at the time of purification of the enzyme, when the observation was made that many of the known substrates of P450 2D6 contain a basic nitrogen atom (*9*, *10*). Inspection of these ligands indicated that the basic nitrogen could be placed 5−7 Å away from the site of oxidation (*10*). The pharmacophore model was further developed for substrates (*12*−*14*) and, by our own group in collaborations, for inhibitors (*11*). Subsequently, Asp301 has been considered to be the putative negative charge bonding to the basic nitrogen, on the combined basis of work with homology modeling and site-directed mutagenesis (*15*−*19*, *52*). These pharmacophore and protein models have been used to rationalize a number of P450 2D6 reactions. The set of ligands used in modeling efforts has been largely restricted to basic amines.

Several major concerns about the use of P450 2D6 models can be raised. First, the models have almost always been used retrospectively and have limitations in the prediction of reactions a priori. This deficiency was found in the work of Grace et al. (*22*) with deprenyl, and other examples (amiflamine, amitryptiline, imipramine) were cited in the same year by Coutts et al. (*23*). Models were subsequently expanded (*24*). The finding of four products of P450 2D6 bufuralol oxidation (*35*, *53*, *54*) emphasizes the problem of predictive capability, in that any model must be flexible enough to accommodate all of these possibilities but then fails to predict specific ones [e.g., the classic substrate debrisoquine yields five oxidation products (*16*)]. Further, predicting the course of the different possible reactions is difficult if not still impossible. The second problem is that the identification of Asp301 as a point charge involved in ionic pairing is compromised by the results of site-directed mutagenesis. The possibility was originally raised that the loss of activity in the Asp301 mutants could also be attributed to a structural effect (*15*) but was discarded in light of the results of heme modification studies (*55*). Our own recent heterologous expression results (*35*) provide support for a major role of Asp301 in structural integrity, not simply one of binding positively charged ligands.

In light of these issues, the existence of high-affinity P450 2D6 ligands devoid of basic nitrogen was considered. Some studies with steroids, particularly testosterone, have yielded K_m or K_i values in the range of 20−30 μM for P450 2D6 (*28*−*30*), which are of the same order of magnitude as many of the basic substrates used in development of the modeling and require consideration. One candidate ligand was spirosulfonamide, which had been discovered as a potential inhibitor of cyclooxygenase-2 (Scheme 1). The phenylsulfonamide of spirosulfonamide contains the only nitrogen in the molecule, and at physiologically relevant pH, this moiety is not positively charged. The sulfonamide moiety is not basic due to the strong electron-withdrawing properties of the sulfone group.[3] The transformations of spirosulfonamide were elucidated using rat hepatocytes, and incubations with microsomes were used to generate sufficient quantities of the major metabolite for characterization. NMR data showed that the oxidation had occurred on the outer methylene group (Scheme 1 and Supporting Information). Experiments using genetically engineered P450s and inhibition in human liver microsomes indicated that P450 2D6 plays a significant role in the oxidation (vide supra) although other enzymes, such as P450 3A4, also contribute.[4]

Further work with spirosulfonamide showed that it produced very typical type I difference spectra, indicative of a low- to high-spin transition due to displacement of H_2O as

[3] The reported pK_a values for sulfonamides are in the range of 9.5−10 in H_2O for an alkyl sulfonamide (*56*) and ~16 in DMSO for a phenyl sulfonamide (*57*). It should be noted that these pK_a values are for the process involving *loss* of a proton from NH_2 ($-SO_2NH_2 \leftrightarrow -SO_2NH^- + H^+$). The protonation of sulfonamides ($-SO_2NH_2 + H^+ \leftrightarrow -SO_2NH_3^+$) has not been studied as much. A reasonable estimate of the pK_a for this process, more relevant to the issue of P450 2D6 pharmacophores, can be made from the known pK_a values for the protonation of alkyl and aryl amides in H_2O, −0.5 to −1.5 (*58*−*60*). The pK_a of an aryl sulfonamide should be much lower due to resonance considerations; i.e., sulfonic acids have much lower pK_a values (<-2.5) (*58*) than the corresponding carboxylic acids, and the pK_a for the equilibrium under consideration would be expected to be <-6.

the distal ligand (*45*), with $K_s = 1.6$ μM and $\Delta A_{max} \sim 0.01$ μM^{-1} (Figure 2). Spirosulfonamide appears to be a reasonably good P450 2D6 substrate, in comparison to others, with a k_{cat} of 6.5 min^{-1} and K_m of 7 μM ($k_{cat}/K_m \sim 2 \times 10^4$ M^{-1} s^{-1}). Asp301 does not appear to contribute directly to binding, as judged by the results of titrations with mutants (Table 1). However, these mutants show attenuated catalytic activity for the oxidation of spirosulfonamide, an uncharged substrate, that parallels the loss of activity seen with the amine substrate bufuralol (*35*) (Table 1). These results argue that the loss of catalytic activity of the Asp301 neutral mutants cannot be attributed to a loss of binding affinity for a cationic substrate, because the same pattern is observed with an uncharged substrate.

The possibility can be considered that Asp301 interacts with spirosulfonamide through hydrogen bonding to the sulfonamide group, as opposed to electrostatic interactions. The methyl sulfone, **2**, totally devoid of nitrogen atoms, had an order of magnitude less affinity than spirosulfonamide (Table 1) and was oxidized less rapidly ($\sim$0.3 min^{-1}), although the same major product was formed. The analogous amide (**3**) and thioamide (**4**) were strong ligands. Intuitively, one might expect less rotation of the amide/thioamide than the sulfonamide and sulfone. Complete removal of the moiety (and the fluorine on the adjacent phenyl ring) yields a compound (**6**) that is both a reasonably tightly bound ligand (K_d 4 μM, Table 1) and a substrate (Figure 6). It is of interest to note that the presence of a carboxylate (**5**) leads to a loss of apparent binding (Table 2) and oxidation. This piece of information, along with the changes in binding (Table 2) and oxidation (Figure 6) with substitution at the phenyl 4-position, indicates that this part of the molecule does interact with the enzyme. Although a clash of the ionized carboxylate (of **5**) with Asp301 could be proposed, there is no evidence to support this view.

Ritonavir (Scheme 2) has two amide linkages and a carbamate, and these might be capable of hydrogen bonding with Asp301 or another entity. However, **6** and the steroid testosterone have only a single (conjugated) carbonyl; progesterone has two carbonyls. All of these compounds bind to P450 2D6 with affinities in the range of many of the amines used in modeling work and are also substrates for P450 2D6. Affinities of some of the strong inhibitors have been measured (*11*), but affinities have not actually been compiled for substrates in the major modeling exercises reported to date (e.g., refs *16*, *18*, and *24*). In general, considerations of P450 2D6 have been largely biased in terms of amine substrates and probably need to be expanded in light of recent information.

The results with non-amine substrates suggest reevaluation of the role of Asp301. We propose that Asp301 plays an important structural role in P450 2D6 integrity. Initial efforts to reverse the putative Asp301−basic substrate interaction with a Lys/Arg301−acidic substrate pair were unsuccessful due to failure of mutants substituted with basic residues at codon 301 to incorporate heme (*35*). Even neutral residues affected heme incorporation and, by inference, protein folding (*35*). Thus, the results of Mackman et al. (*55*) on heme modification of Asp301 mutants notwithstanding, we conclude that mutations of Asp301 cause more extensive changes in P450 2D6 than can be interpreted in the context of electrostatic interaction with ligands (although hydrogen bonding etc. could not be completely ruled out). In this regard, the same patterns of loss of catalytic activity are seen (Table 3) with the mutants for both a protonated substrate (bufuralol) and an unprotonated (neutral) substrate (spirosulfonamide), even under conditions where the ligand concentration (30 μM) is near saturating for all mutants (Table 3). In other studies leading to this work, we have found that some phenethylamine substrates for P450 2D6 show both type I and type II spectra that are pH-dependent, and P450 2D6 can be characterized as changing the pK_a of the ligand, reducing it 1−3 pH units (*63*). These results indicate the existence of a residue with a pK_a of $\sim$6.6, which would be unusual for Asp although not impossible. However, any such acid−base chemistry is probably not relevant to spirosulfonamide binding.[3]

We have not investigated the roles of Phe481 and Phe483, suggested by others' models (*24*, *26*), in the binding of spirosulfonamide; evidence has been presented that these residues do not explain the ability of P450 2D6 to catalyze amine N-dealkylation (*25*). Our current view of the substrate binding site of P450 2D6 is that several residues probably contribute direct ligand contacts or more global effects on structure. The importance of distant parts of a P450 (P450 101) on ligand binding has been discussed recently by Gray and associates (*64*). We cannot rule out possibilities of "shaping" of the active site by ligands through an induced-fit mechanism, whether involving local or distant parts of P450 2D6.

In conclusion, these results clearly show the lack of a requirement for a basic nitrogen atom for high-affinity binding. The possibility exists that some substrates may use a negative charge (e.g., Asp301) in their binding interactions, either through the use of an electrostatic interaction or H-bonding, and others not. However, prediction of new P450 2D6 ligands and their oxidation sites, based on the premise that a basic nitrogen is required, is compromised by the existence of uncharged, non-nitrogen-containing ligands with high affinity. The existing pharmacophore and homology models of P450 2D6 have been used to rationalize binding and oxidation of some ligands, but the predictive usefulness of these models is questionable, as is borne out in some studies of known substrates (e.g., refs *16*, *22*, *23*, and *35*). The conclusions described herein, based on the results of Asp301 site-directed mutagenesis (*35*) and the finding of high-affinity ligands and substrates devoid of basic, positively charged nitrogen atoms (Scheme 2), demonstrate that our understanding of the P450 2D6 active site is unsound and that further modeling regarding the role of Asp301 is required.

[4] Caution should be exercised in overinterpretation of the preliminary experiments because DMSO was used to prepare the stock solutions of spirosulfonamide; high concentrations of DMSO are known to have inhibitory effects on catalytic activities of some P450s (*61*, *62*) although DMSO was not an issue in another study (*47*).

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Syntheses of spirosulfonamide, *syn*- and *anti*-hydroxyspirosulfonamide, and keto spirosulfonamide; biosynthesis and mass and NMR spectral characterization of the oxidation products of spirosulfonamide (**1**) and spiromethyl sulfone (**2**); and Dynafit scripts for using DynaFit to estimate competition of ligands with bufuralol for P450 2D6. This material is available free of charge via the Internet at http://pubs.acs.org.

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