Interaction of Sulfaphenazole Derivatives with Human Liver Cytochromes P450 2C: Molecular Origin of the Specific Inhibitory Effects of Sulfaphenazole on CYP 2C9 and Consequences for the Substrate Binding Site Topology of CYP 2C9

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ABSTRACT: The effects of sulfaphenazole, **1**, on typical activities catalyzed by human cytochromes P450 of the 1A, 3A, and 2C subfamilies expressed in yeast were studied. **1** acts as a strong, competitive inhibitor of CYP 2C9 ($K_i = 0.3 \pm 0.1$ μM); it is much less potent toward CYP 2C8 and 2C18 ($K_i = 63$ and 29 μM, respectively) and fails to inhibit CYP 1A1, 1A2, 3A4, and 2C19. From difference visible spectroscopy experiments using microsomes of yeast expressing various human P450s, **1** selectively interacts only with CYP 2C9 with the appearance of a peak at 429 nm as expected for the formation of a P450 Fe(III)−nitrogenous ligand complex ($K_s = 0.4 \pm 0.1$ μM). Comparative studies of the spectral interaction and inhibitory effects of twelve compounds related to **1** with CYP 2C9 showed that the aniline function of **1** is responsible for the formation of the iron−nitrogen bond of the 429 nm-absorbing complex and is necessary for the inhibitory effects of **1**. The study of two new compounds synthesized during this work, in which the *N*-phenyl group of **1** was replaced with either an ethyl group or a 3,4-dichlorophenyl group, showed that the presence of an hydrophobic substituent at position **1** of the pyrazole function of **1** is required for a strong interaction with CYP 2C9. A model for the binding of **1** in the CYP 2C9 active site is proposed; that takes into account three major interactions that should be at the origin of the high-affinity and specific inhibitory effects of **1** toward CYP 2C9: (i) the binding of its nitrogen atom to CYP 2C9 iron, (ii) an ionic interaction of its SO_2N^- anionic site with a cationic residue of CYP 2C9, and (iii) an interaction of its *N*-phenyl group with an hydrophobic part of the protein active site.

The oxidative metabolism of drugs and other xenobiotics in man is in great part under the control of cytochrome P450-dependent monooxygenases. In order to interpret or to predict various problems that may occur with some drugs in relation to genetic polymorphism and drug−drug interactions, it is crucial to determine as soon as possible which human liver cytochrome(s) P450 is (are) mainly involved in the metabolism of a given drug. This requires simultaneous approaches using hepatocytes, recombinant enzymes and human liver microsomes in the presence of specific inhibitors of the various human P450s. P450s of the 3A and 2C subfamilies are the major isoforms present in human liver (Guengerich & Turvy, 1991; Guengerich, 1995). Moreover, they are, with CYP[1] 2D6, the P450s most often encountered in the metabolism of pharmaceuticals in humans (Smith et al., 1992). Sulfaphenazole, **1**, a sulfamide drug (Figure 3), is considered as a selective inhibitor of CYP 2C9 (Correia, 1995), the member of the CYP 2C subfamily that is expressed at the highest level in human liver (Ged et al., 1988; Romkes et al., 1991; Goldstein et al., 1994). The inhibitory effects of sulfaphenazole on the *in vivo* metabolism of tolbutamide have been shown by several authors (Christensen et al., 1963; Schulz & Schmidt, 1970; Rowland et al., 1974; Pond et al., 1977; Veronese et al., 1990). Such inhibitory effects on tolbutamide hydroxylation have been shown *in vitro* either on human liver microsomes (Back et al., 1988; Miners et al., 1988) or on recombinant CYP 2C9 (Brian et al., 1989a). It is only very recently that sulfaphenazole was shown to fail to inhibit specific activities of various P450s different from CYP 2C9 in human liver microsomes (1A2, 2A6, 2B6, 2C19, 2D6, 2E1, 3A4, and 4A) (Baldwin et al., 1995; Newton et al., 1995). The corresponding results have been obtained by following the oxidation by human liver microsomes of specific substrates of these isoforms. However, despite the reputation of sulfaphenazole as a specific inhibitor of CYP 2C9, data have not yet been published on its direct effects on the various members of the human liver CYP 2C subfamily nor on the molecular mechanism of its inhibitory effects.

This paper reports the results of a study of the spectral and inhibitory effects of sulfaphenazole on several recombinant human liver cytochromes P450 expressed in yeast, including the four members of the CYP 2C subfamily present in human liver, CYP 2C8, 2C9, 2C18, and 2C19. Sulfaphenazole is shown to be a very selective inhibitor of CYP 2C9 ($K_i \approx 0.3$ μM) and acts as a ligand of high-affinity for CYP 2C9 iron ($K_s \approx 0.4$ μM). A detailed comparison of

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[1] Abbreviations: CYP or P450, cytochrome P450; DMF, dimethylformamide; DTP, 2,3-dichloro-4-(2-thenoyl)phenol.

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many derivatives of sulfaphenazole, some of which have been prepared for this study, allowed us to understand the structural factors that are important for sulfaphenazole recognition by the CYP 2C9 active site.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals used were of the highest quality commercially available. Sulfaphenazole was a gift of Ciba-Geigy (Basel, Switzerland). Tienilic acid was provided by Anphar-Rolland (Chilly-Mazarin, France). Carbutamide was provided by Eli Lilly (Indianapolis, IN). Sulfadiazine and sulfabenzamide were purchased from Sigma, and sulfisoxazole from Janssen. Amicon Kieselgel 60 (35−70 mesh) was used for column chromatography. Recombinant bacterial P450 2C9 was prepared and purified as described elsewhere (Sandhu et al., 1993).

Physical Measurements. UV−visible spectra were performed on a Kontron Uvikon 820 spectrophotometer equipped with a diffusion sphere. ^{1}H NMR spectra were recorded at 27 °C on a Bruker ARX-250 instrument; chemical shifts are reported downfield from $(CH_3)_4Si$ and coupling constants are in Hz. The following abbreviations, s, d, t, m, bs, and dd, are used for singlet, doublet, triplet, multiplet, broad singlet, and doublet of doublet, respectively. Mass spectra (MS) were performed with chemical ionization (CI) using NH_3 on a Nermag R1010 apparatus. For compound **5** that contains two chlorines atoms, all peaks corresponding to molecular ions or fragments involving two chlorine exhibited the isotope cluster shape expected for the presence of two Cl atoms (with an [M]:[M+2]:[M+4] ratio of 10:6:1); *m/z* indicated in the following are for ^{35}Cl. Elemental analyses were carried out at Centre Regional de Microanalyse, Paris.

Synthesis of Sulfaphenazole Derivatives

*5-(p-Acetamidobenzenesulfonamido)-1-phenylpyrazole, **2***. Sulfaphenazole (0.5 mmol) was dissolved in glacial acetic acid (3 mL) with heating. Acetic anhydride (0.5 mmol) was added to the solution and after 45 min at 60 °C, the solvent was evaporated and the residue purified by column chromatography (SiO_2, CH_2Cl_2−MeOH 4% as eluent). The product obtained in a quantitative yield was recrystallized from CH_2Cl_2; mp 164−165 °C. ^{1}H NMR (CD_3SOCD_3): 10.32 and 10.26 (2 bs, 2H, 2NH), 7.70 (d, 2H, J = 8.9), 7.58 (d, 2H, J = 8,9), 7.53 (d, 1H, J = 1.9), 7.46−7.36 (m, C_6H_5), 5.81 (d, 1H, J = 1.9), 2.09 (s, 3H, CH_3). El. analysis, calcd for $C_{17}H_{16}N_4O_3S$: C, 57.29; H,4.53; N, 15.72. Found: C, 57.30; H, 4.52; N, 15.85.

*5-Methanesulfonamido-1-phenylpyrazole, **3***. To an ice cold solution of 5-amino-1-phenylpyrazole (160 mg, 1 mmol) in $CHCl_3$ (2 mL) were added pyridine (100 μL) and methanesulfonyl chloride (80 μL, 1 mmol). After 2 h at room temperature and 18 h at 40 °C, the reaction mixture was washed with 0.1 N HCl, extracted with CH_2Cl_2, and dried over $MgSO_4$. Compound **3** was purified by column chromatography (SiO_2, ethyl acetate−cyclohexane 1/1 as eluent) and obtained as an oil in 60% yield. ^{1}H NMR ($CDCl_3$): 2.90 (s, 3H, CH_3), 6.38 (d, 1H, J = 1.9), 6.43 (bs, 1H, NH), 7.48 (m, 5H, Ph), 7.62 (d, 1H, J = 1.9). El. analysis, calcd for $C_{10}H_{11}N_3O_2S$: C, 50.62; H, 4.67; N, 17.71. Found: C, 50.71; H, 4.88; N, 17.84.

*5-(p-Methylbenzyl)amino-1-phenylpyrazole, **4***. The Schiff base was prepared by heating 5-amino-1-phenylpyrazole (160 mg) and *p*-methylbenzaldehyde (120 μL) in the absence of solvent at 100 °C for 15 min, and the *N*-benzylidene amine was purified by column chromatography (SiO_2, CH_2Cl_2−acetone 100:7.5) (70 mg, 27% yield). To a solution of the above Schiff base (66 mg, 0.25 mmol) in methanol (0.5 mL) warmed at 40 °C was added sodium borohydride (10 mg); a steady evolution of hydrogen occurred. Then the solution was heated under reflux for further 15 min. 1 mL H_2O was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$. The amino compound was purified by column chromatography (SiO_2, CH_2Cl_2−acetone 100:7.5) which led to 60 mg of **4** as colorless oil (90% yield). ^{1}H NMR ($CDCl_3$): 2.33 (s, 3H, CH_3), 4.07 (bt, 1H, NH), 4.23 (d, 2H, J = 6), 5.54 (d, 1H, J = 2), 7.14 (d, 2H, J = 8), 7.22 (d, 2H, J = 8), 7.31 (t, 1H, J = 7.6), 7.44 (m, 3H), 7.55 (d, 2H, J = 7.6). El. analysis, calcd for $C_{17}H_{17}N_3$: C, 77.54; H, 6.51; N, 15.96. Found: C, 77.53; H, 6.47; N, 15.91.

*5-(p-Aminobenzenesulfonamido)-1-(m,p-dichlorophenyl)-pyrazole, **5***. Synthesis of 5-(*p*-aminobenzenesulfonamido)-1-(*m*,*p*-dichlorophenyl)pyrazole, **5**, was performed according to a synthetic strategy already described for sulfaphenazole (Schmidt & Druey, 1958). Acrylonitrile (1.5 mL) and 3,4-dichlorophenylhydrazine (3.6 g) in 20 mL of EtOH were heated under reflux for 15 h, and the solvent was then evaporated under vacuum and the residue purified by column chromatography (SiO_2, CH_2Cl_2) to yield 2.44 g of *N*-dichlorophenyl-*N′*-(β-cyanoethyl)hydrazine (mp 69−70 °C). ^{1}H NMR ($CDCl_3$): 2.56 (t, 2H, J = 6.5, CH_2CN), 3.15 (t, 2H, J = 6.5, CH_2NH), 3.90 (bs, 1H, NH), 5.17 (bs, 1H, NH), 6.70 (dd, 1H, J = 2.7, J = 8.7), 7.01 (d, 1H, J = 2.7), 7.22 (d, 1H, J = 8.7). A solution of this product (1.15 g) in 9 mL of 2 N H_2SO_4 was stirred 17 h at room temperature with 2 g of anhydrous $Fe_2(SO_4)_3$. The mixture was extracted with Et_2O. After drying over Na_2SO_4 and evaporation of the solvent, the residue was purified by column chromatography (SiO_2, CH_2Cl_2). After boiling of a 1 N NaOH solution of the obtained azo compound (800 mg in 11 mL of 1 N NaOH), extraction with CH_2Cl_2, and recrystallization from CH_2Cl_2, 780 mg of 5-amino-1-(*m*,*p*-dichlorophenyl)pyrazole were obtained (98% yield); mp 141−142 °C. ^{1}H NMR ($CDCl_3$): 3.78 (bs, 2H, NH2), 5.63 (d, 1H, J = 1.9), 7.54−7.41 (m, 3H), 7.77 (d, 1H, J = 1.9). El. analysis, calcd for $C_9H_7Cl_2N_3$: C, 47.40; H, 3.09; N, 18.42. Found: C, 47.51; H, 3.06; N, 18.39.

4-Acetamidobenzenesulfonyl chloride (130 mg, 0.55 mmol) was added to a solution of 5-amino-1-(*m*,*p*-dichlorophenyl)-pyrazole (114 mg, 0.5 mmol) in anhydrous pyridine (1 mL). After 1 h at 95 °C, the solvent was evaporated and the residue partially dissolved in CH_2Cl_2 was washed with 0.1N HCl. Extraction with CH_2Cl_2 removed the unreacted amine and extraction with ethyl acetate gave the desired acetamide of **5**. Recrystallization from acetone−CH_2Cl_2 gave 93 mg of crystalline product (44% yield); mp 199−200 °C. ^{1}H NMR (CD_3COCD_3): 2.11 (s, 3H, CH_3), 6.03 (d, 1H, J = 1.9), 7.52−7.27 (m, 6H), 7.62 (d, 1H, J = 8,8), 8.65 and 9.24 (2bs, 2 NH).

After treatment of the acetamide of **5** (87 mg, 0.2 mmol) with boiling 2 N NaOH (2 mL) for 2 h, the reaction mixture was adjusted to pH 4 with HCl, extracted with CH_2Cl_2, and dried over $MgSO_4$. Compound **5** was purified by column chromatography (SiO_2; CH_2Cl_2−acetone 10:0.75) and recrystallized from ether−cyclohexane (66% yield); mp 149−

150 °C. ^{1}H NMR ($CDCl_3$): 4.19 (bs, 2H, NH_2), 6.20 (d, 1H, $J = 1.9$), 6.30 (bs, 1H, NH), 6.55 (d, 2H, $J = 8.7$), 7.20 (dd, 1H, $J = 8.7$ and 2.4), 7.34 (d, 1H, $J = 2.4$), 7.40 (d, 2H, $J = 8.7$), 7.44(d, 1H, $J = 8.7$), 7.55 (d, 1H, $J = 1.9$). El. analysis,calcd for $C_{15}H_{12}Cl_2N_4O_2S$: C, 47.01; H, 3.16; N, 14.62. Found: C, 47.51; H, 3.12; N, 14.06. MS (CI, NH_3): $m/z = 383$ ([M + H$^+$], 100%); 228 (55%); 194 (38%); 156 (17%).

5-(p-Acetamidobenzenesulfonamido)-1-ethylpyrazole. N-Acetylsulfanilyl chloride (270 mg, 1.15 mmol) was added to a solution of 5-amino-1-ethylpyrazole (100 mg, 0.9 mmol) in anhydrous pyridine (1 mL). After 1 h at 95 °C, the solvent was evaporated and the residue purified by column chromatography (SiO_2, CH_2Cl_2 and then 5% MeOH as eluent). Traces of pyridine were removed by washing the product dissolved in ethyl acetate with 10^{-3} M HCl. A white solid (230 mg, 83% yield) was obtained; mp 124−125 °C. ^{1}H NMR (CD_3SOCD_3): 10.33 and 10.14 (2 bs, 2H, 2NH), 7.75 (d, 2H, $J = 8.8$), 7.64 (d, 2H, $J = 8.8$), 7.27 (d, 1H, $J = 1.8$), 5.56 (d, 1H, $J = 1.8$), 3.92 (q, 2H, $J = 7.2$, CH_2), 2.07 (s, CH_3CO), 1.16 (t, 3H, $J = 7.2$, CH_3). MS (CI, NH_3): $m/z = 309$ ([M+H]$^+$, 15%); 136 (17%); 112 (100%).

5-(p-Aminobenzenesulfonamido)-1-ethylpyrazole, 6. 5-(*p*-Acetamidobenzenesulfonamido)-1-ethylpyrazole (83 mg, 0.27 mmol) was treated with 2 mL of 2 N NaOH under reflux for 2 h. Then the reaction mixture was adjusted to pH 5−6 with 4 N HCl and filtered, and the product was crystallized from EtOH (97% yield); mp 193−194 °C. ^{1}H NMR (CD_3SOCD_3): 9.7 (bs, 1H, NH), 7.32 (d, 2H, $J = 8.7$), 7.25 (d, 1H, $J = 1.8$), 6.57 (d, 2H, $J = 8.7$), 6.02 (s, 2H, NH_2), 5.57 (d, 1H, $J = 1.8$), 3.91 (q, 2H, $J = 7.2$, CH_2), 1.16 (t, 3H, $J = 7.2$, CH_3). MS (CI, NH3): $m/z = 267$ ([M+H]$^+$, 34%); 186 (58%); 126 (23%); 112 (100%). El. analysis, calcd for $C_{11}H_{14}N_4O_2S$: C, 49.61; H, 5.30; N, 21.04. Found: C, 49.55; H, 5.25; N, 21.13.

Determination of the pK_a Value of ***1***. The pK_a value of sulfaphenazole was determined by a classical acid−base titration method (Alexander & Griffith, 1993) using a micro pH 2001 (Grison) pH meter. This value, p$K_a = 6.0$, is in agreement with that reported previously (Bell & Roblin, 1942).

Yeast Transformation, Cell Culture, and Preparation of Yeast Microsomal Fraction. Two yeast systems have been used for CYP 2C9 expression. The first one used yeast strain D12 [a, leu$^-$, (cir$^+$)], and the human cDNA MP-4 (2C9) clone, previously reported (Umbenhauer et al., 1987; Ged et al., 1988; Brian et al., 1989b), was inserted in the pAAH5 vector. Details of protocols for pAAH5/MP-4 vector construction and yeast transformation and screening are presented elsewhere (Brian et al., 1989a,b; Srivastava et al., 1991). Yeast microsomes were prepared as previously described (Brian et al., 1989b; Guengerich et al., 1991) using yeast lytic enzyme (Sigma) for cell wall digestion followed by sonication. The final microsomal pellet was homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v), aliquoted, frozen under liquid N_2, and stored at −80 °C until use. Usual expression levels of CYP 2C9 were 1 nmol of P450/L of culture, and microsomes contained 60 pmol of P450/mg of protein.

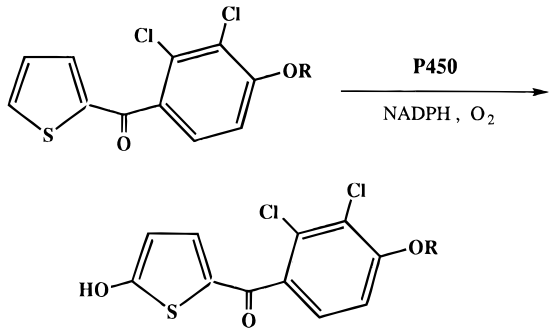
The second expression system was used not only for expression of CYP 2C9 but also for human liver CYP 1A1, 1A2, 2C8, 2C18, 2C19, and 3A4. It is based on a yeast strain W(R) fur1 previously described (Truan et al., 1993), in which yeast cytochrome P450 reductase was overexpressed after transformation by a pYe DP60 vector containing one of the CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19 and 3A4 cDNAs (Gautier et al., 1993; Lecoeur et al., 1994; Renaud et al., 1993), according to a general method of construction of yeast strain W(R) fur1 expressing various human liver P450s (Urban et al., 1994). Yeast culture and microsomes preparation were performed by using previously described techniques (Bellamine et al., 1994). Microsomes were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v), aliquoted, frozen under liquid N_2, and stored at −80 °C until use. P450 contents of yeast microsomes were 200, 100, 40, 90, 40, 20, and 200 pmol of P450/mg of protein for CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, and 3A4, respectively.

Microsomal P450 content was determined according to Omura and Sato (1964). The protein content in microsomal suspensions was determined by the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as standard.

Study of Substrate Binding to Yeast-Expressed Human CYP Forms by Difference Visible Spectroscopy. Yeast microsomes were suspended in 50 mM Tris/HCl, 1 mM EDTA, pH 7.4, to obtain a P450 concentration of about 150−200 nM. The solution was equally divided between both cuvettes of a Kontron 820 spectrophotometer. After the base line was recorded, aliquots (1−5 μL) of solutions containing the studied compound were added to the sample cuvette, the same volume of solvent being added to the reference cuvette. After 3 min, the difference spectra were recorded between 360 and 520 nm. Measurements were carried at 20 °C.

Enzyme Activity Assays

5-Hydroxylation of 2-Aroylthiophenes. 5-Hydroxylation of tienilic acid (eq 1) was followed as a reference activity



R = CH_2COOH **tienilic acid**

R = H **2,3-dichloro-4(2-thenoyl)phenol, DTP**

for CYP 2C9 and CYP 2C18 (Lopez-Garcia et al., 1993), whereas the 5-hydroxylation of its phenol analog DTP (eq 1) was studied in the case of CYP 2C8 and CYP 2C19. Quantitation of 5-hydroxy-2-aroylthiophenes was based on a spectrophotometric method (Neau et al., 1990) adapted to yeast microsomes expressing P450 2C9 in the case of tienilic acid (Lopez-Garcia et al., 1993). The conditions used in this study of hydroxylation of tienilic acid and DTP were previously described (Mancy et al., 1995). Inhibition studies of the 5-hydroxylation of 2-aroylthiophenes were performed at various concentrations of inhibitor (1−500 μM) and four concentrations of substrate in the range 10−100 μM. The inhibitor and the substrate were added simultaneously to the

Table 1: Inhibition by Sulfaphenazole of Different Activities Specifically Catalyzed by Yeast-Expressed Human Liver Cytochromes P450[a]

CYP	activity	inhibition[b]	K_i (μM)
1A1	ethoxyresorufin-*O*-deethylation	−	
1A2	ethoxyresorufin-*O*-deethylation	−	
3A4	testosterone 6β-hydroxylation	−	
2C8	DTP-5-hydroxylation	+	63 ± 4
2C9	tienilic acid 5-hydroxylation	+	0.3 ± 0.1
2C18	tienilic acid 5-hydroxylation	+	29 ± 3
2C19	DTP-5-hydroxylation	−	

[a] Initial rates were measured after a 10 min incubation of yeast microsomes W(R) fur1 expressing CYP 1A1, 1A2, 3A4, 2C8, 2C9, 2C18, and 2C19 (0.2 μM) in the presence of 100 μM of substrate (200 μM of substrate was used for ethoxyresorufin) and a NADPH-generating system. Initial rates in nmol of product (nmol of P450)$^{-1}$ min^{-1} were 10, 1, 1.8, 0.6, 1.9, 1.8, and 0.8 in the presence of yeast microsomes expressing CYP 1A1, 1A2, 3A4, 2C8, 2C9, 2C18, and 2C19, respectively. [b] −, no significant inhibitory effects even with 500 μM sulfaphenazole. Values are the mean ± SD from three to four experiments.

incubation mixture. K_i values were derived from the analyses of Lineweaver−Burk plots corresponding to the various enzymatic activities in the presence of increasing concentrations of sulfaphenazole. Curve fitting was performed by a single regression fit procedure of Cricket Graph (Cricket Software, Malvern, PA) on a Macintosh personal computer (Lopez-Garcia et al., 1994).

6β-Hydroxylation of Testosterone. The assay for testosterone 6β-hydroxylation (Brian et al., 1990) was carried out using 100 μM testosterone. The reaction proceeded for 10 min at 28 °C.

Ethoxyresorufin-O-deethylation. The 7-ethoxyresorufin-O-deethylase (EROD) activity was measured at 28 °C in yeast microsomes using a previously described method (Prough et al., 1978).

RESULTS AND DISCUSSION

Effects of Sulfaphenazole on Different Recombinant Human Liver Cytochromes P450. The effects of sulfaphenazole on typical activities catalyzed by human P450s of the 1A, 3A, and 2C subfamilies expressed in yeast were studied. Yeasts obtained by transformation of yeast strain W(R) fur1, in which cytochrome P450 reductase was overexpressed (Truan et al., 1993), with a pYeDP60 vector containing the cDNA of a given human P450, were used (Urban et al., 1994). Ethoxyresorufin-*O*-deethylation by microsomes from yeast producing CYP 1A1 or 1A2 was followed as a classical reaction of cytochrome P450 of the 1A subfamily (Prough et al., 1978), whereas 6β-hydroxylation of testosterone was followed in the case of CYP 3A4 (Brian et al., 1990), 5-hydroxylation of tienilic acid in the case of CYP 2C9 and CYP 2C18 (Lopez-Garcia et al., 1993; Lecoeur et al., 1994), and 5-hydroxylation of a phenol analog of tienilic acid, 2,3-dichloro-4-(2-thenoyl)phenol, DTP (eq 1) (Mancy et al., 1995), in the case of CYP 2C8 and CYP 2C19. As shown in Table 1, sulfaphenazole failed to inhibit the activities catalyzed by microsomes of yeasts expressing CYP 1A1, 1A2, 3A4, and 2C19, even at concentrations as high as 500 μM. It acted as a very strong competitive inhibitor of CYP 2C9-dependent 5-hydroxylation of tienilic acid (Figure 1), with a K_i value of 0.3 ± 0.1 μM. It is noteworthy that an identical K_i value was obtained (0.3 ± 0.1 μM, data not shown) for the inhibition of tienilic acid 5-hydroxylation by microsomes from another yeast strain D12 transformed with the pAAH5 vector containing CYP 2C9 cDNA (Brian et al., 1989b). Experiments similar to those whose results are shown in Figure 1 established that sulfaphenazole also acted as a competitive inhibitor of CYP 2C18 and CYP 2C8 expressed in yeast (data not shown). However, the corresponding K_i values were much higher, i.e., 63 ± 4 μM and 29 ± 3 μM for CYP 2C8 and CYP 2C18, respectively (Table 1). The following experiments have been done to understand the origin of this particularly strong inhibitory effects of sulfaphenazole toward CYP 2C9.

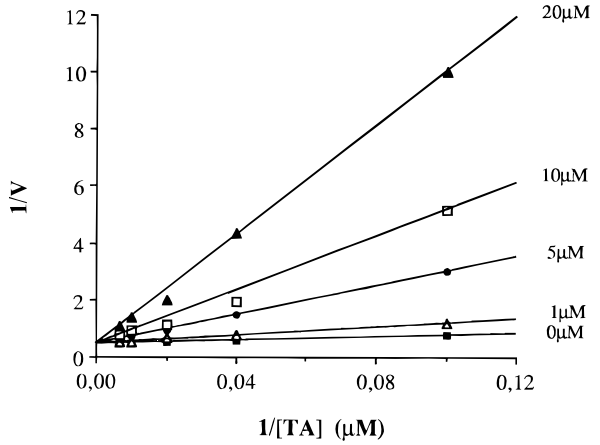


FIGURE 1: Inhibition of recombinant CYP 2C9-dependent 5-hydroxylation of tienilic acid by sulfaphenazole. Plots of the reciprocal of initial 5-hydroxytienilic acid formation rates vs 1/[TA] (TA = tienilic acid) in the presence of various sulfaphenazole concentrations (1, 5, 10, 20 μM). Rates in nmol of 5-hydroxytienilic acid product (nmol of P450)$^{-1}$ min^{-1} were measured after a 10 min incubation of yeast microsomes W(R) fur1 yeast expressing CYP 2C9 (0.2 μM P450) in 0.1 M phosphate buffer, pH 7.4, in the presence of 10−100 μM tienilic acid with various sulfaphenazole concentrations (0−20 μM) and a NADPH-generating system.

Spectral Interaction of Sulfaphenazole with Recombinant Human P450s. Addition of sulfaphenazole to microsomes of CYP 2C9-expressing yeast led to the appearance of a difference visible spectrum characterized by a peak at 429 nm and a trough at 395 nm (Figure 2), which is typical of P450−Fe(III) complexes with nitrogenous ligands. The apparent spectral dissociation constant, K_s, that was deduced from the increase of intensity of this spectrum as a function of sulfaphenazole concentration, was found to be 0.4 ± 0.1 μM (Table 2). This value is almost identical to the K_i value found previously (Table 1), indicating that the inhibitory effects of that compound toward CYP 2C9 should result from its binding as an iron ligand of this cytochrome. Here again, identical spectral results were obtained with microsomes either from yeast strain W(R) fur1 or from yeast strain D12 producing CYP 2C9 (data not shown). Similar spectral results were obtained with recombinant CYP 2C9 purified either from yeast or *Escherichia coli* expressing this cytochrome (Sandhu et al., 1993).

Interestingly, interaction of sulfaphenazole (at concentrations up to 500 μM) with microsomes from yeast strain W(R) fur1 expressing CYP 1A1, 1A2, 3A4, 2C8, 2C18, or 2C19 failed to give any significant difference visible spectrum.

Interaction of Sulfaphenazole Derivatives with CYP 2C9

Mode of Binding of Sulfaphenazole to CYP 2C9 Iron. A priori, three nitrogen atoms of sulfaphenazole, **1**, may bind

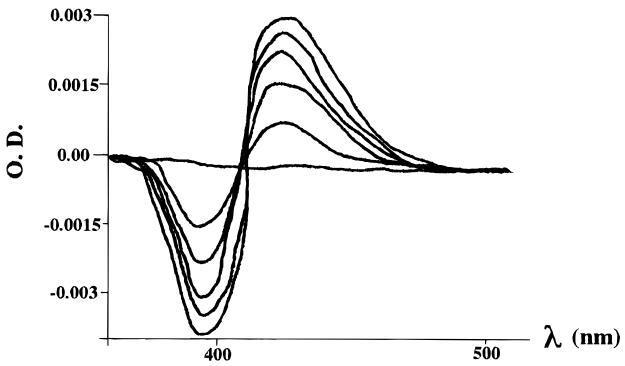


FIGURE 2: Difference visible spectra obtained upon addition of sulfaphenazole to microsomes of W(R) fur1 yeast expressing CYP 2C9. Yeast microsomes W(R) fur1 expressing CYP 2C9 were suspended in 50 mM Tris HCl, 1 mM EDTA, pH 7.4, to obtain a P450 concentration of 0.2 μM and the solution (1 mL) was equally divided between both cuvettes. After the base line was recorded, aliquots (0.5−5 μL) of a sulfaphenazole solution were added to the sample cuvette, the same volume of solvent (methanol) being added to the reference cuvette. A 0−10 μM sulfaphenazole concentration range was thus covered. Measurements were carried at 20 °C.

Table 2: Effects of Various Analogs of Sulfaphenazole on the Visible Spectrum and Tienilic 5-Hydroxylation Activity of Microsomes from Yeast-Expressing CYP 2C9

compounds	$\Delta A_{429-395}$[a] ($\times 10^3$)	K_s[b] (μM)	IC_{50}[c]
sulfaphenazole			
1	7	0.4 ± 0.1	0.8 ± 0.1
2	−		>500[d]
3	−		>500[d]
4	−		>500[d]
5	7	0.1 ± 0.03	0.5 ± 0.1
6	−		>500[d]
sulfisoxazole	−		>500[d]
sulfadiazine	−		>500[d]
sulfabenzamide	−		>500[d]
carbutamide	−		>500[d]

[a] $\Delta A_{429-395}$ are maximum absorbances obtained in difference visible spectroscopy for the type II spectrum appearing after addition of saturing concentrations of the compound to a suspension of microsomes from W(R) fur1 yeast containing 0.2 μM P450 in Tris HCl 50mM pH 7.4 (1 cm path length cuvette). (−), no spectral difference detectable upon compound addition ($\Delta A < 0.1 \times 10^{-3}$). [b] Apparent dissociation constants (K_s) were calculated from the reciprocal plot of the absorbance change versus inhibitor concentration. [c] IC_{50} values were measured by using increasing concentrations of potential inhibitors added to the assay incubation mixture including CYP 2C9 microsomes (0.2 μM P450) in phosphate buffer, pH 7.4, 10 μM tienilic acid, and a NADPH-generating system. [d] No significant inhibitory effects even with 500 μM compound. Values are means ± SD from four experiments.

to CYP 2C9−Fe(III) to lead to the 429 nm absorbing complex shown in Figure 2. They come from the NH_2, $NHSO_2$ and pyrazole (nonsubstituted N) functions of sulfaphenazole (Figure 3). In order to distinguish between these three possibilities, compound **2** in which the NH_2 function of **1** was acetylated, and derivatives **3** and **4** were synthesized. The latter were prepared from reaction of 5-amino-1-phenylpyrazole with appropriate alkyl or sulfonyl chlorides (Figure 4). Their spectral interaction with CYP 2C9 was studied as well as that of 1-phenylpyrazole and 5-amino-1-phenylpyrazole. The two latter compounds did not yield any difference spectra with microsomes from yeast expressing CYP 2C9, indicating that their pyrazole nitrogen cannot bind to CYP 2C9. In fact, compounds **3** and **4**, in which the aniline function was absent, and *N*-acetylsulfaphenazole, **2**, were unable to give any difference spectrum with microsomes from yeast expressing CYP 2C9 (Table 2). These data indicate that the type II difference spectrum observed with **1** (Table 2) is due to the binding of its NH_2 group to CYP 2C9 iron.

*Importance of the Phenylpyrazole Moiety in the Affinity of **1** for CYP 2C9.* As the aniline function of **1** appeared crucial for the binding of **1** to CYP 2C9, interaction of this cytochrome with several compounds containing an aniline function was studied, in order to determine the structural factors that are responsible for the strong binding of **1**. Already described compounds involving the $NH_2C_6H_4SO_2$-NH moiety of **1** were studied first, including carbutamide, an analog of a known CYP 2C9 substrate, tolbutamide (Brian et al., 1989a; Goldstein et al., 1994), in which the $CH_3C_6H_4$ group was replaced by an aniline function (Figure 3). Thus, in that compound, the CH_3 group of tolbutamide, the site of hydroxylation, is replaced by an NH_2, which might be expected to bind to the heme iron. Other compounds, sulfadiazine, sulfisoxazole, and sulfabenzamide, which respectively contain a pyrimidine, isoxazole, and benzoyl group instead of the phenylpyrazole group of **1**, were also studied. None of them led to any difference spectrum with CYP 2C9 nor acted as inhibitors of CYP 2C9-dependent 5-hydroxylation of tienilic acid (even at concentrations up to 500 μM) (Table 2). These results clearly showed that the phenylpyrazole moiety of **1** is crucial for the high affinity of **1** for CYP 2C9. Then, two analogs of **1** in which the phenyl substituent of **1** was replaced with either an ethyl or a 3,4-dichlorophenyl group were synthesized and tested. The method used for their synthesis is illustrated in Figure 4 in the case of the ethyl derivative **6**. The analog of sulfaphenazole with a dichlorophenyl group, **5**, exhibited a behavior similar to that of **1**, as it gave a 429 nm-absorbing complex with CYP 2C9 and strongly inhibited CYP 2C9-dependent 5-hydroxylation of tienilic acid (Table 2). Interestingly, its K_s value was even slightly lower than that of **1** (0.1 ± 0.03 μM). On the contrary, the N-ethyl analog **6** was found to be unable to inhibit CYP 2C9 and to produce a difference spectrum (even at concentrations as high as 500 μM) (Table 2). This result demonstrates the key role of the substituent at position **1** of the pyrazole ring in the recognition of **1** by the CYP 2C9 active site.

A Possible Model for the Interaction of Sulfaphenazole with the CYP 2C9 Active Site. Taken altogether, the aforementioned results show that the very high affinity of **1** for CYP 2C9 is based on at least two interactions: (i) the binding of its NH_2 nitrogen atom to CYP 2C9 iron, which is responsible for the appearence of the observed difference spectrum, and (ii) strong hydrophobic (or stacking) bonds between its *N*-phenyl group and amino acid residue(s) of CYP 2C9 active site. Quite recently, a model for the substrate binding site of CYP 2C9 has been reported [Mancy et al., 1995; for other models, see also B. C. Jones et al. (1996) and J. P. Jones et al. (1996)]. In this model, the substrates of CYP 2C9, which most often are anionic at pH 7.4, interact through their anionic site with a cationic residue of the distal side of the protein. Sulfaphenazole **1** contains a SO_2NH function whose pK_a is 6.0 (Bell & Roblin, 1942). It thus mainly exists as an anion at pH 7.4 and could establish a third kind of bond with CYP 2C9, an electrostatic interaction between its SO_2N^- site and the putative cationic residue of the protein implicated in substrate binding. Recent studies of the interaction of several substrates with recom-

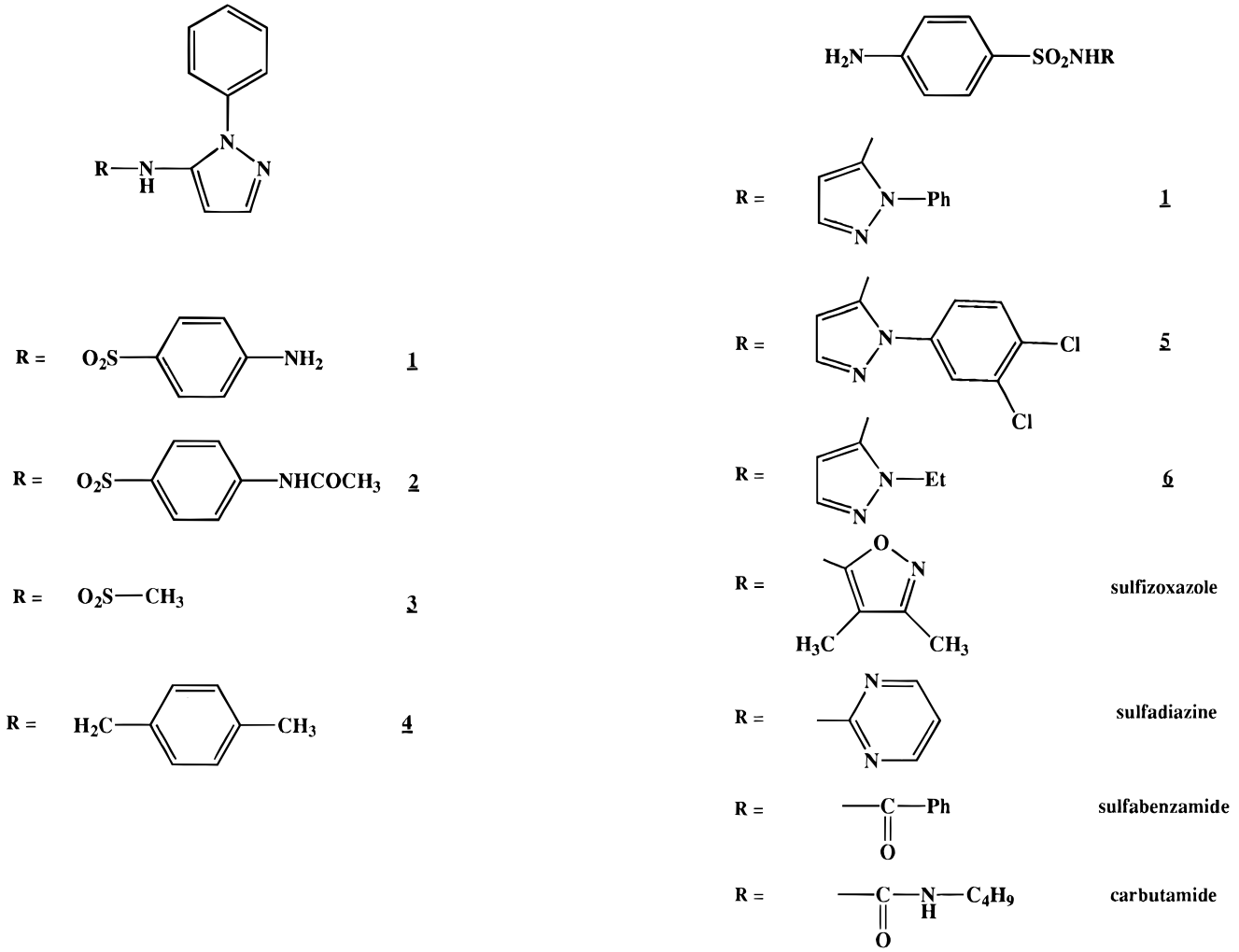


FIGURE 3: Structure of sulfaphenazole, **1**, and of its derivatives used in this study.

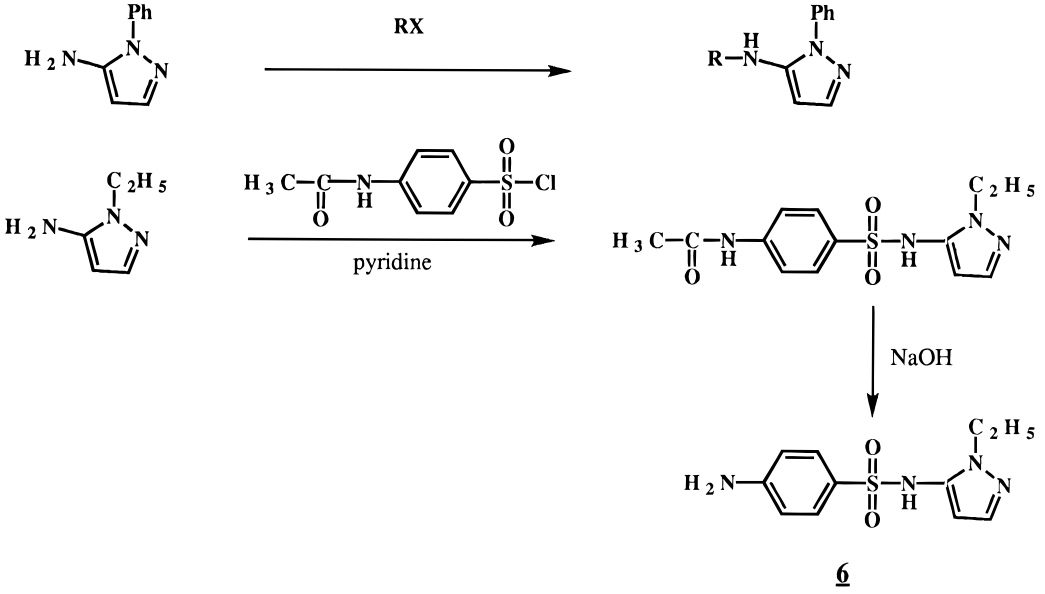


FIGURE 4: Schematic routes used for the synthesis of sulfaphenazole analogs.

binant CYP 2C9 by paramagnetic relaxation effects on the ^{1}H NMR spectrum of these substrates allowed us to calculate distances between the heme iron atom and substrate protons and to determine the orientation of the substrates relative to CYP 2C9 iron (S. Poli, R. Attias, P. Dansette, D. Mansuy, in preparation). Figure 5 shows the positioning of a good substrate of CYP 2C9, tienilic acid, relative to the heme iron atom, in that model. Molecular modeling calculations (Insight and Discover softwares from MSI-Biosym) were thus made to incorporate sulfaphenazole in that set of substrates in CYP 2C9 active site. The conformation of **1** corresponding to its X-ray structure (Patel & Singh, 1987)

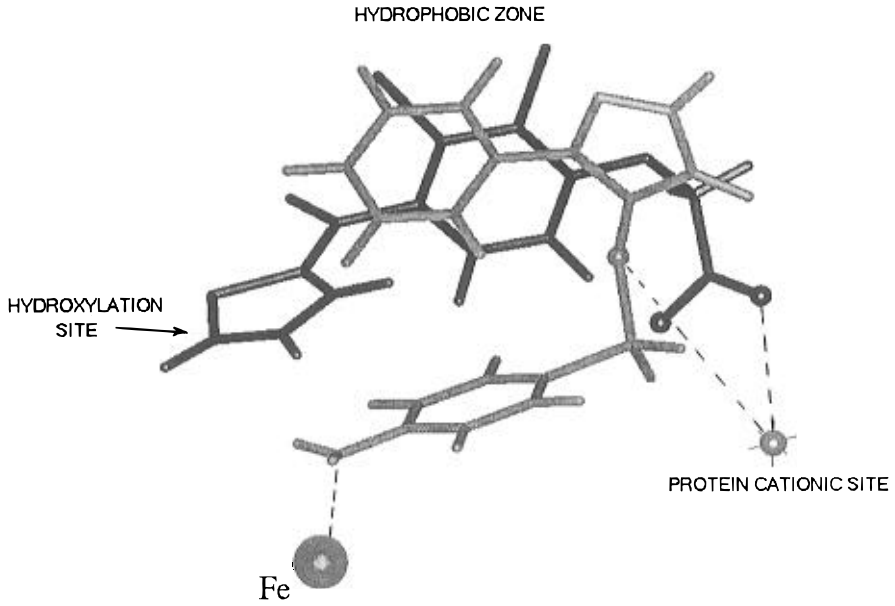


FIGURE 5: Possible mode of interaction of sulfaphenazole with CYP 2C9. Tienilic acid (in black) is shown as a representative of the CYP 2C9 substrate envelope recently proposed (Mancy et al., 1995), that has been positioned relative to CYP 2C9 iron after ^{1}H NMR experiments (see text). Sulfaphenazole **1** (in grey) was incorporated in that model after taking into account its X-ray structure and its binding to CYP 2C9 iron *via* its NH_2 function. Interaction of the anionic groups of **1** and tienilic acid with a putative cationic amino acid residue of CYP 2C9 active site is also shown.

was used initially. **1** was overlayed onto CYP 2C9 substrates such as phenytoin and tienilic acid that were used to build the substrate envelope described previously (Mancy et al., 1995). Its NH_2 nitrogen atom was positioned 2.2 Å from CYP 2C9 iron, a classical value for (porphyrin) iron−nitrogen distances in iron−primary alkylamine complexes (Mansuy et al., 1983). It was possible to find a conformation of **1** leading to such a Fe−N bond in which the N^- anionic site of **1** was located at a distance from the protein cationic site compatible with an ionic bond (3.7 Å) (Figure 5). This conformation was only 4 kcal higher than the minimum energy of **1**. Interestingly, in that conformation, the *N*-phenyl group of **1** is very well located in the hydrophobic region of the CYP 2C9 substrate envelope (close to the hydrophobic aryl group of tienilic acid for instance, Figure 5).

The above data indicate that it is possible to include **1** in the model of substrate active site of CYP 2C9 previously proposed (Mancy et al., 1995). The corresponding positioning of **1** takes into account (i) the binding of its NH_2 nitrogen atom to CYP 2C9 iron, (ii) an electrostatic interaction of its SO_2N^- anionic site with a cationic residue of CYP 2C9, and (iii) an interaction of its *N*-phenyl residue with an hydrophobic part of the protein active site. This last interaction should be strong as replacement of the *N*-phenyl group with an ethyl substituent dramatically decreased the affinity of **1** for CYP 2C9 (Table 2). It could be an hydrophobic or/and a $\pi-\pi$ interaction with aromatic amino acid residues of the protein. Replacement of the phenyl residue of **1** with a 3,4-dichlorophenyl group improves its affinity for CYP 2C9 (Table 2).

The very high affinity of **1** for CYP 2C9 ($K_s \approx 0.4 \pm 0.1$ μM, $K_i \approx 0.3 \pm 0.1$ μM) and its great selectivity for this isozyme (Table 1) should come from the conjunction of those three strong interactions. This is also true for the new compound **5** which exhibits the highest affinity for CYP 2C9 ($K_s \approx 0.1$ μM) and appears to be the best inhibitor of this enzyme (Table 2).

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REFERENCES

Alexander, R. R., & Griffiths, J. M. (1993) in *Basic Biochemical Methods*, pp 7−16, Wiley-Liss, New York.
Back, D. J., Tjia, J. F., Karbwang, J., & Colbert, J. (1988) *Br. J. Clin. Pharmacol. 26*, 23−29.
Baldwin, S. J., Bloomer, J. C., Smith, G. J., Ayrton, A. D., Clarke, S. E., & Chenery, R. J. (1995) *Xenobiotica 25*, 261−270.
Bell, P. H., & Roblin, R. O. (1942) *J. Am. Chem. Soc. 64*, 2905−2911.
Bellamine, A., Gautier, J. C., Urban, P., & Pompon, D. (1994) *Eur. J. Biochem. 225*, 1005−1013.
Brian, W. R., Srivastava, P. K., Umbenhauer, D. R., Lloyd, R. S., & Guengerich, F. P. (1989a) *Biochemistry 28*, 4993−4999.
Brian, W. R., Ged, C., Bellew, T. M., Srivastava, P. K., Bork, R. W., Umbenhauer, D. R., Lloyd, R. S., & Guengerich, F. P. (1989b) *Drug Metab. Rev. 20*, 449−465.
Brian, W. R., Sari, M. A., Iwasaki, M., Shimada, T., Kaminsky, L., & Guengerich, F. P. (1990) *Biochemistry 29*, 11280−11292.
Christensen, L. K., Hansen, J. M., & Kristensen, M. (1963) *Lancet ii*, 1298−1301.
Correia, M. A. (1995) in *Cytochrome P450, Structure, Mechanism and Biochemistry*, pp 607−630, Plenum Press, New York.
Gautier, J. C., Urban, P., Beaune, P., & Pompon, D. (1993) *Eur. J. Biochem. 211*, 63−72.
Ged, C., Umbenauer, D. R., Bellew, T. M., Bork, R. W., Srivastava, P. K., Shinriki, N., Lloyd, R. S., & Guengerich, F. P. (1988) *Biochemistry 27*, 6929−6940.
Goldstein, J. A., Faletto, M. B., Romkes-Sparks, M., Sullivan, T., Kitareewan, S., Raucy, J. L., Lasker, J. M., & Ghanayem, B. I. (1994) *Biochemistry 33*, 1743−1752.
Guengerich, F. P., & Turvy, C. G. (1991) *J. Pharmacol. Exp. Ther. 256*, 1189−1194.
Guengerich, F. P., Brian, W. R., Sari, M. A., & Ross, J. T. (1991) *Methods Enzymol. 206*, 130−145.

Guengerich, F. P. (1995) in *Cytochrome P450, Structure, Mechanism and Biochemistry*, pp 473−515, Plenum Press, New York.
Jones, B. C., Hawksworth, G., Horne, V. A., Newlands, A., Morsman, J., Tute, M. S., & Smith, D. A.(1996) *Drug Metab. Dispos. 24*, 260−266.
Jones, J. P., He, M., Trager, W. F., & Rettie, A. E. (1996) *Drug Metab. Dispos. 24*, 1−6.
Lecoeur, S., Bonierbale, E., Challine, D., Gautier, J. C., Valadon, P., Dansette, P. M., Catinot, R., Ballet, F., Mansuy, D., & Beaune, P. H. (1994) *Chem. Res. Toxicol. 7*, 434−442.
Lopez Garcia, M. P., Dansette, P. M., Valadon, P., Amar, C., Beaune, P. H., Guengerich, F. P., & Mansuy, D. (1993) *Eur. J. Biochem. 213*, 223−232.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Mancy, A., Broto, P., Dijols, S., Dansette, P. M., & Mansuy, D. (1995) *Biochemistry 34*, 10365−10375.
Mansuy, D., Battioni, P., Chottard, J.-C., Riche, C., & Chiaroni, A. (1983) *J. Am. Chem. Soc. 105*, 455−463.
Miners, J. O., Smith, K. J., Robson, R. A., McManus, M. E., Veronese, M. E., & Birkett, D. J. (1988) *Biochem. Pharmacol. 37*, 1137−1144.
Neau, E., Dansette, P. M., Andronik, V., & Mansuy, D. (1990) *Biochem. Pharmacol. 39*, 1101−1107.
Newton, D. J., Wang, R. W., & Lu, A. Y. H. (1995) *Drug Metab. Dispos. 23*, 154−158.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370−2378.
Patel, H. C., & Singh, T. P. (1987) *Acta Crystallogr. C43*, 1131−1134.
Pond, S. M., Birkett, D. J., & Wade, D. N. (1977) *Clin. Pharmacol. Ther. 22*, 573−579.
Prough, R. A., Burke, M. D., & Mayer, R. T. (1978) *Methods Enzymol. 52 LII* (39), 372−377.
Renaud, J. P., Peyronneau, M. A., Urban, P., Truan, G., Cullin, C., Pompon, D., Beaune, P., & Mansuy, D. (1993) *Toxicology 82*, 39−52.
Romkes, M., Faletto, M. B., Blaisdell, J. A., Raucy, J. L., & Goldstein, J. A. (1991) *Biochemistry 30*, 3247−3255.
Rowland, M., Matin, S. B., Thiessen, J., & Karam, J. (1974) in *Kinetics of Tolbutamide Interactions*, pp 199−210, Raven, New York.
Sandha, P., Baba, T., & Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 306*, 443−450.
Schmidt, P., & Druey, J. (1958) *Helv. Chim. Acta 41*, 306−309.
Schulz, E., & Schmidt, F. A. (1970) *Pharmacol. Clin. 2*, 150−153.
Smith, D., A., & Jones, B. C. (1992) *Biochem. Pharmacol. 44*, 2089−2098.
Srivastava, P. K., Yun, C. H., Beaune, P. H., Ged, C., & Guengerich, F. P. (1991) *Mol. Pharmacol. 40*, 69−79.
Truan, G., Cullin, C., Reisdorf, P., Urban, P., & Pompon, D. (1993) *Gene 125*, 49−55.
Umbenhauer, D. R., Martin, M. V., Lloyd, R. S., & Guengerich, F. P. (1987) *Biochemistry 26*, 1094−1099.
Urban, P., Truan, G., Bellamine, A., Laine, R., Gautier, J. C., & Pompon, D. (1994) *Drug Metab. Drug Interact. 11*, 169−200.
Veronese, M. E., Miners, J. O., Randles, D., Gregov, D., & Birkett, D. J. (1990) *Clin. Pharmacol. Therap. 47*, 403−411.

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