The Substrate Binding Site of Human Liver Cytochrome P450 2C9: An Approach Using Designed Tienilic Acid Derivatives and Molecular Modeling†

Annah Mancy,‡ Pierre Broto,§ Sylvie Dijols,‡ Patrick M. Dansette,‡ and Daniel Mansuy*,‡

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, URA 400 CNRS, Université Paris V, 45 Rue des Saints-Pères, 75270 Paris Cedex 06, France, and Roussel-Uclaf, Direction des Recherches, 102 Route de Noisy, 93230 Romainville, France

Received March 20, 1995; Revised Manuscript Received May 15, 1995⊗

ABSTRACT: Biochemical experiments, using the well-defined human liver CYP2C9 expressed in yeast, and molecular modeling techniques were used to derive a predictive model for substrates of CYP2C9. The ability of 10 2-aroylthiophenes related to tienilic acid to act as substrates for CYP2C9 was studied. Four of them were original compounds that were synthesized and completely characterized by several spectroscopic techniques. In these 10 compounds various chemical functions, such as ester, amide, alcohol, phenol, ether or tetrazole functions, replaced the OCH_2COOH function of tienilic acid. Among them, only the derivatives containing an acidic function (carboxylic acids, phenol, and tetrazole whose pK_as are 4.8, 6.3, and 3.8, respectively) underwent a 5-hydroxylation of their thiophene ring like tienilic acid. Despite their close structural analogy with tienilic acid, all of the other compounds not only did not undergo any 5-hydroxylation of their thiophene ring but also failed to act as inhibitors of CYP2C9. These results strongly suggested that the presence, at pH 7.4, of a negative charge on the substrate is a very important feature in its recognition by CYP2C9. In fact, the four new substrates of CYP2C9 described in this study, a carboxylic acid, phenol, and tetrazole derivative, each of which is related to tienilic acid, and the antiinflammatory drug, suprofen (with K_m between 12 and 130 μM and k_{cat} between 0.2 and 1.3 min^{-1}), as well as almost all CYP2C9 substrates reported in the literature, exhibit a pK_a below 7 (except phenytoin whose pK_a is 8.1). They mainly exist as anions at physiological pH. By using molecular modeling techniques, 12 CYP2C9 substrates were superimposed with respect to their hydroxylation site and fitted onto templates, which were rigid molecules such as (*S*)-warfarin and phenytoin. It was thus possible to arrange them in order that all their anionic sites were at a distance around 4 Å from a common point (a putative cationic site of the protein) in space. These results provide a model of the substrate binding site of CYP2C9, in which substrates interact through their anionic site A^- with a cationic residue of the CYP2C9 protein C^+. In that model, the distance between the hydroxylation site (Hy) and the anionic site (A^-) is 7.8 ± 1.6 Å, and the $\angle HyA^-C^+$ angle is 82 ± 15°.

P450[1] enzymes play a key role in the metabolism of exogenous compounds such as drugs. Their broad substrate specificity is now well understood on the basis of enzyme multiplicity (Gonzalez, 1989). So far, several hundreds of P450s have been sequenced and classified on the basis of their amino acid sequence similarity (Nelson et al., 1993). P450s of the 3A and 2C subfamilies are the major isoforms present in human liver (Guengerich & Turvy, 1991). Moreover, they are the isoforms most often encountered in the metabolism of pharmaceuticals in humans. Genetic analysis of the CYP2C subfamily indicates the presence of at least seven genes with several allelic variants. From the main members of this subfamily known so far, namely, CYP2C8, -2C9, -2C18, and -2C19, CYP2C9 seems to be the protein expressed at the highest level in human liver (Ged et al., 1988; Romkes et al., 1991). CYP2C9 has been expressed in COS (Veronese et al., 1991) and Hep G2 (Aoyama et al., 1990) cells, as well as in *Escherichia coli* (Sandhu et al., 1993) and yeast (Brian et al., 1989a; Ohgiya et al., 1989; Yasumori et al., 1989). The encoded protein has been found able to catalyze the hydroxylation of tolbutamide (Brian et al., 1989a; Doecke et al., 1991; Goldstein et al., 1994a; Relling et al., 1990; Srivastava et al., 1991), phenytoin (Doecke et al., 1991; Veronese et al., 1991), (*S*)-warfarin (Rettie et al., 1992), hexobarbital (Brian et al., 1989a; Sandhu et al., 1993), and tienilic acid (Lopez-Garcia et al., 1993). A purified CYP2C9 has been found to be able to catalyze the hydroxylation of a quinone-containing drug, 58C80 (Weaver et al., 1993). Moreover, subsequent to experiments performed on human liver microsomes and using inhibitors of CYP2C9, it has been proposed that certain nonsteroidal antiinflammatory drugs such as tenoxicam, piroxicam (Zhao et al., 1992), naproxen (Newlands et al., 1992), ibuprofen (Leemann et al., 1993b, 1994), and diclofenac (Leemann et al., 1993a) could be substrates of CYP2C9. Therefore, this P450 isoform could play a very important role in drug interactions.

Studies described in this paper have been performed to determine the nature of the substrate binding site of CYP2C9.

† This work was supported by a grant from the Ministère de la Recherche et de la Technologie and Rhône-Poulenc (Programme Bioavenir) and a fellowship to A.M. from Roussel-Uclaf.

* To whom correspondence should be addressed. FAX: 33-1-42 86 83 87.

‡ Université Paris V.

§ Roussel-Uclaf.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1995.

[1] Abbreviations: CYP or P450, cytochrome P450; DMF, dimethylformamide; NSAIDs, nonsteroidal antiinflammatory drugs.

0006-2960/95/0434-10365$09.00/0

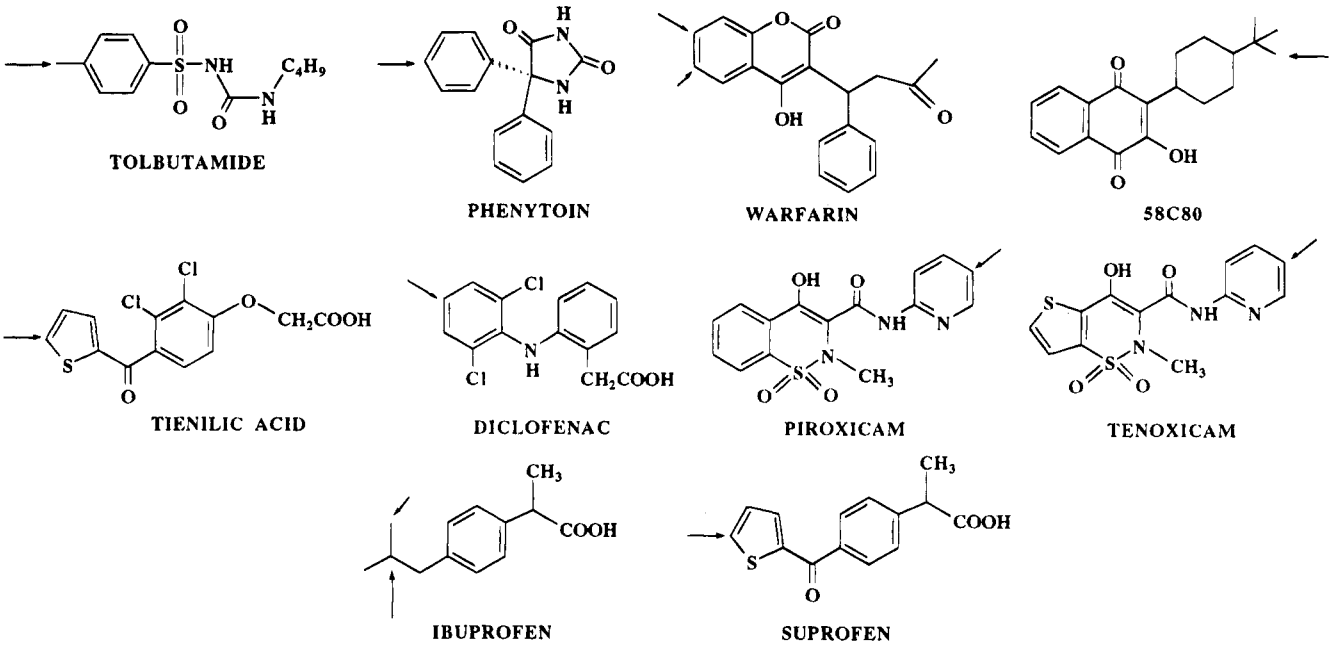


FIGURE 1: Structures of the main substrates described so far for CYP2C9 (the ability of suprofen to act as a CYP2C9 substrate was shown in this paper). Arrows indicate the site(s) of hydroxylation by CYP2C9.

In this regard it is noteworthy that all the aforementioned substrates of CYP2C9 are protic acids with a pK_a between 4.5 and 8.1 (Figure 1 and Table 1). They are either carboxylic acids (diclofenac, ibuprofen, and tienilic acid) or compounds containing an acidic NH group bound to $ArSO_2$ and/or COR electron-withdrawing residues (phenytoin, tolbutamide) or an especially acidic phenol function (warfarin, piroxicam, tenoxicam, and 58C80). These substrates mainly exist as anions at physiological pH, and it is tempting to speculate that the substrate binding site of CYP2C9 involves an amino acid residue that could be responsible for recognition of substrates by ionic or hydrogen-bonding interactions (Jones et al., 1994). Three series of experiments have been undertaken to address this question and to explore the topology of the CYP2C9 substrate binding site. The first one addresses the ability of a series of compounds related to tienilic acid (**1**), one of the best substrates known of CYP2C9 (Table 1), to act as substrates of CYP2C9 expressed in yeast. Several tienilic acid derivatives whose carboxylic acid group was replaced by various chemical functions have been synthesized for that purpose. In the second series of experiments, the ability of several compounds including drugs to inhibit yeast-expressed CYP2C9 was studied. On the basis of the biochemical results of these two series of experiments and of molecular modeling studies made on the conformations of the substrates of CYP2C9 known so far, a model for the CYP2C9 substrate binding site is proposed.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest quality commercially available. Tienilic acid was provided by Anphar-Rolland (Chilly-Mazarin, France). Sulfaphenazole was a gift of Ciba-Geigy (Basel, Switzerland). Diclofenac, naproxen, tolbutamide, ibuprofen, ketoprofen, phenytoin, piroxicam, and warfarin were purchased from Sigma. Suprofen was purchased from Janssen. Silica gel from Amicon (Kieselgel 60, 35−70 mesh) was used for column chromatography.

Physical Measurements. UV−visible spectroscopy was 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 J are in hertz. Labeling of the carbons of 2-aroylthiophenes corresponding to the following H assignments is given in Figure 2. Mass spectral (MS) analysis was performed with chemical ionization (CI) using NH_3 on a Nermag R1010 apparatus. For all compounds related to tienilic acid that contain two chlorine atoms, all peaks corresponding to molecular ions or fragments involving two Cl exhibited the isotope cluster shape expected for the presence of two Cl atoms (with a 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 Tienilic Acid Derivatives. Tienilic acid methyl ester was prepared by a previously described method (Levron et al., 1977; Mansuy et al., 1984).

*4-(2-Thenoyl)phenoxyacetic Acid (**8**).* Compound **8** was prepared according to a synthetic strategy already described for tienilic acid (Herbert & Pichat, 1976). The last step is the reaction of 4-(2-thenoyl)phenol (35 mg) in 2 mL of anhydrous DMF in the presence of NaH (30 mg) with sodium chloroacetate (40 mg). After 2 h reaction at 100 °C, careful hydrolysis of excess NaH, and addition of 1 N HCl to pH ~2, the reaction mixture was extracted with diethyl ether. Purification by thin-layer chromatography (silica gel, elution with toluene−ethyl acetate−acetic acid 15:60:5 v/v/v) led to a new product (R_f = 0.31). Dissolution of this product in 0.1 N ammonia and extraction of the impurities by ethyl acetate led, after lyophilization, to compound **8** in a 33% yield. ^{1}H NMR (CD_3SOCD_3): 4.67 (s, 2H, CH_2), 7.26 (t, 1H, J = 5), 7.70 (d, 1H, J = 5), 8.04 (d, 1H, J = 5), 7.79 (d, 2H, J = 9), 6.92 (d, 2H, J = 9). MS (CI, NH_3) m/z = 263 ($[M + H]^+$, 100%), 280 ($[M + NH_4]^+$, 65%).

*2,3-Dichloro-4-(2-thenoyl)phenol (**5**).* Compound **5** and its methoxy ether precursor **6** were prepared by a previously

described procedure (Neau et al., 1990). Their ^{1}H NMR spectra were in complete agreement with those reported previously (Neau et al., 1990).

*2-[2,3-Dichloro-4-(2-thenoyl)phenoxy]ethanol (**4**).* 2-Chloroethanol (60 μL, 0.6 mmol) was added to a solution of **5** (55 mg, 0.2 mmol) in anhydrous DMF (0.5 mL) containing 0.2 mmol of K_2CO_3. After 24 h at 80 °C, DMF was evaporated and the residue dissolved in CH_2Cl_2. The organic phase was washed with acidic water and dried over $MgSO_4$. Compound **4** was purified by column chromatography (SiO_2, CH_2Cl_2 as eluent) and obtained as white crystals in a 72% yield (after purification): mp = 117−118 °C. ^{1}H NMR ($CDCl_3$): 2.10 (t, 1H, OH, J = 6.4), 4.03 (m, 2H, CH_2O), 4.20 (t, 2H, CH_2OAr, J = 4.8), 6.92 (d, 1H, $H_{5'}$, J = 8.6), 7.11 (dd, 1H, H_4, $J_{4,3}$ = 4, $J_{4,5}$ = 4.8), 7.33 (d, 1H, $H_{6'}$, J = 8.6), 7.41 (dd, 1H, H_3, $J_{3,4}$ = 4, $J_{3,5}$ = 1.2), 7.74 (dd, 1H, H_5, $J_{5,4}$ = 4.8, $J_{5,3}$ = 1.2). Anal. Calcd for $C_{13}H_{10}Cl_2O_3S$: C, 49.23; H, 3.18. Found: C, 49.27; H, 3.19. MS (CI, NH_3) m/z = 317 ($M + H^+$, 100), 272 ($[M - CH_2CH_2O]^+$, 4), 189 ($C_7H_3Cl_2O_2^+$, 6), 111 ($C_5H_3OS^+$, 28).

*2,3-Dichloro-4-(2-thenoyl)phenoxyacetamide (**3**).* Iodoacetamide (37 mg, 0.2 mmol) was added to a solution of **5** (55 mg, 0.2 mmol) in 1 mL of anhydrous DMF containing 30 mg of K_2CO_3. After 3 h at 80 °C, evaporation of DMF, extraction with CH_2Cl_2, and purification by column chromatography (SiO_2, CH_2Cl_2:diethyl ether = 95:5), compound **3** was obtained as white crystals, mp = 165−166 °C, in a 98% yield. ^{1}H NMR ($CDCl_3$): 4.61 (s, 2H, CH_2), 5.74 and 6.65 (bs, 2H, $CONH_2$), 6.96 (d, 1H, $H_{5'}$, J = 8.6), 7.15 (dd, 1H, H_4, $J_{4,3}$ = 4, $J_{4,5}$ = 4.8), 7.41 (m, 2H, H_3 and $H_{6'}$), 7.81 (dd, 1H, H_5, $J_{5,4}$ = 4.8, $J_{5,3}$ = 1.2). Anal. Calcd for $C_{13}H_9Cl_2NO_3S$: C, 47.29; H, 2.75; N, 4.24. Found: C, 47.13; H, 2.87; N, 4.17. MS (CI, NH_3) m/z = 347 ($[M + NH_4]^+$, 100), 330 ($M + H^+$, 73), 296 ($[M - Cl + 2H]^+$, 31), 111 ($C_5H_3OS^+$, 60).

2,3-Dichloro-4-(2-thenoyl)phenoxyacetonitrile. Chloroacetonitrile (0.2 mmol) was added to a solution of **5** (55 mg, 0.2 mmol) in 1 mL of anhydrous DMF containing 30 mg of K_2CO_3. After 2 h at 70 °C and workup similar to that used for the aforementioned products, the nitrile was obtained as white crystals, mp = 116−117 °C, in a 98% yield. ^{1}H NMR ($CDCl_3$): 4.91 (s, 2H, CH_2), 7.06 (d, 1H, $H_{5'}$, J = 8.6), 7.12 (dd, 1H, H_4, $J_{4,3}$ = 4, $J_{4,5}$ = 4.8), 7.40 (m, 2H, H_3 and $H_{6'}$), 7.76 (d, 1H, H_5, $J_{5,4}$ = 4.8). Anal. Calcd for $C_{13}H_7Cl_2NO_2S$: C, 50.02; H, 2.26; N, 4.49. Found: C, 50.11; H, 2.24; N, 4.51. MS (CI, NH_3) m/z = 329 ($[M + NH_4]^+$, 53), 312 ($M + H^+$, 100), 111 ($C_5H_3OS^+$, 44).

*[[2,3-Dichloro-4-(2-thenoyl)phenoxy]methyl]tetrazole (**9**).* Preparation of **9** was carried out according to a general procedure for the synthesis of tetrazoles (Kadaba, 1973). A mixture of the above nitrile (0.32 mmol), sodium azide (0.35 mmol), and ammonium chloride (0.35 mmol) in anhydrous DMF (0.5 mL) was heated at 120 °C for 5 h. After removal of DMF, the reaction mixture was dissolved in 0.5 mL of H_2O and made acidic with HCl (pH 2). This step must be performed carefully as free HN_3 evolved. After cooling to 5 °C, the precipitated tetrazole was removed by filtration, washed with cold water, dried, and recrystallized from CH_2Cl_2: mp = 197−198 °C (75% yield). ^{1}H NMR (CD_3OD): 5.65 (s, 2H, CH_2), 7.19 (t, 1H, H_4, J = 4), 7.35 (d, 1H, $H_{5'}$, J = 8.8), 7.46 (m, 2H, H_3 and $H_{6'}$), 7.98 (d, 1H, H_5, J = 4.8). Anal. Calcd for $C_{13}H_8Cl_2N_4O_2S$: C, 43.96; H, 2.27; N, 15.77. Found: C, 44.07; H, 2.31; N, 15.92. MS (CI, NH_3) m/z = 372 ($[M + NH_4]^+$, 100), 355 ($M + H^+$, 35), 273 ($[M + H - C_2H_2N_4]^+$, 32).

Determination of pK_a Values. The pK_a values of suprofen and compounds **1**, **5**, **8**, and **9** were determined by a classical acid−base titration method (Alexander & Griffith, 1993) using a micro pH 2001 (Grison) pH meter. The value found for compound **5** was also obtained from a UV spectroscopy method which took profit of the different UV spectra of the phenol and phenolate forms of **5** (isobestic point at 326 nm for the passage from the acidic form to the phenolate). Both techniques gave a pK_a of 6.3 ± 0.2 for compound **5**. The pK_as of suprofen (4.9 ± 0.2), tienilic acid (4.8 ± 0.2), **8** (4.8 ± 0.2), and **9** (3.8 ± 0.2) are given in the text.

Yeast Transformation, Cell Culture, and Preparation of Yeast Microsomal Fraction. The isolation and sequencing of the human cDNA MP-4(2C9) clone has been previously reported (Umbenhauer et al., 1987; Ged et al., 1988; Brian et al., 1989b). This DNA sequence was inserted in the vector pAAH5 (based on an ADH1 promoter and terminator) which contains the *leu2* gene. *Saccharomyces cerevisae* strain D12 [a, leu$^-$, (cir$^+$)] was used for expression. 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).

The transformed yeasts, referred to in the text as pAAH5/P450 2C9, were cultured semianaerobically at 28 °C in synthetic minimal medium [0.67% (w/w) Difco yeast nitrogen base without amino acids and 2% (w/v) glucose] supplemented with amino acids (except leucine). Growth was monitored carefully by light scattering at 600 nm, and the cultures were allowed to reach an OD_{600} of 1.7−1.8 (about 5 × 10^7 cells/mL), at which time culture was stopped by quick refrigeration. Cells were harvested by centrifugation (5000g, 5 min), pooled, and washed twice with distilled water. Yeast cell pellets were stored at −80 °C until they were processed for microsome preparation.

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.

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. Usual expression levels of CYP2C9 were 1 nmol of P450/L of culture, and microsomes contained 0.07 nmol of P450/mg of protein.

Enzyme Activity Assays. 5-Hydroxylation of 2-Aroylthiophenes. Quantitation of 5-hydroxy-2-aroylthiophenes was based on a spectrophotometric method (Neau et al., 1990) adapted to yeast microsomes expressing CYP2C9 in the case of tienilic acid (Lopez-Garcia et al., 1993). For all 2-aroylthiophenes that were found to be 5-hydroxylated by yeast-expressed CYP2C9, controls were run to show that no hydroxylation occurred in the absence of NADPH or by microsomes from control yeast in the presence of NADPH. The hydroxylation of each substrate by yeast-expressed CYP2C9 was studied as a function of time and was shown to be linear for at least 10 min for all compounds studied. Therefore, the following activities were measured after 10

Table 1: Substrates of CYP2C9: pK_a and Characteristics of the Catalyzed Oxidation[a]

compound	pK_a	type of oxidation	hydroxylation site	K_m (μM)	k_{cat} (min^{-1})	materials used
diclofenac	4.5[b]	aromatic hydroxylation	4′	6		human liver[c]
ibuprofen	4.5[b]	aliphatic hydroxylation	iPr (CH and CH_3)	53		human liver[d]
phenytoin	8.1[e]	aromatic hydroxylation	4	45		COS cell exp[f]
piroxicam	5.7[g]	aromatic hydroxylation	5′	50		human liver[h]
suprofen	4.9[g]	aromatic hydroxylation	5	45	0.9	yeast exp[g]
tenoxicam	5.7[g]	aromatic hydroxylation	5′	40		human liver[h]
tolbutamide	5.4[b]	aliphatic hydroxylation	CH_3	132		COS cell exp[i]
tienilic acid (**1**)	4.8[g]	aromatic hydroxylation	5	6	2.4	yeast exp[j]
(*S*)-warfarin	5.1[k]	aromatic hydroxylation	7	4	0.4	Hep cell exp[l]
			6	4	0.12	Hep cell exp[l]
58C80		aliphatic hydroxylation	CH_3	141	53.8	purified CYP2C9[m]

[a] The indicated data are drawn from the literature except for suprofen, which was studied in this work. Data were obtained by using either human liver microsomes and specific CYP2C9 inhibitors or CYP2C9 expressed in heterologous systems (last column). In the case of 58C80, a purified CYP2C9 was used (Weaver et al., 1993). The hydroxylation site was indicated according to the literature nomenclature for each product; for the sake of clarity, the hydroxylation sites by CYP2C9 were also indicated by arrows in Figure 1. [b] Smith & Jones, 1992. [c] Leemann et al., 1993a. [d] Leemann et al., 1994. [e] Rosenberg & Jackson, 1989. [f] Veronese et al., 1993. [g] This work. [h] Zhao et al., 1992. [i] Veronese et al., 1991. [j] Lopez-Garcia et al., 1993. [k] Opong-Mensah et al., 1984. [l] Rettie et al., 1994. [m] Weaver et al., 1993.

min incubation periods. All conditions were those previously described for the 5-hydroxylation of tienilic acid (Lopez-Garcia et al., 1993).

Inhibition of the Microsomal 5-Hydroxylation of Tienilic Acid by Various Compounds. Inhibition studies of the 5-hydroxylation of tienilic acid were performed at various concentrations of inhibitor (25−1000 μM) and four concentrations of tienilic acid in the range of 10−100 μM. The inhibitor and tienilic acid were added simultaneously to the incubation mixture.

Molecular Modeling. Computer-assisted molecular modeling studies were carried out using a Silicon Graphics Iris 4D/25 work station. Twelve substrates of CYP2C9 were compared. For seven of them (phenytoin, warfarin, tienilic acid, tolbutamide, diclofenac, ibuprofen, and piroxicam), X-ray crystallographic data were available in the literature, and the corresponding conformations were used without further modification. For the other six molecules, the initial conformations were calculated by using the Biosym cvff force field and fully minimized. The final conformation used was kept within an 8 kcal·mol^{-1} range above the energy minimum obtained previously. Two rigid compounds, phenytoin and (*S*)-warfarin, were superimposed with respect to their hydroxylation sites and the aromatic ring bearing the hydroxylation site. Then, all the other substrates hydroxylated at an aromatic ring were overlayed by the same method. It was possible to find an arrangement of all molecules such that their anionic heteroatoms were at a distance of ~4 Å from a common point in the space (C^+ in Figure 6). Finally, the compounds whose hydroxylation site is an aliphatic carbon (tolbutamide, 58C80, ibuprofen) were overlayed on the other molecules with superposition of their hydroxylated carbon. In the arrangement of the 12 molecules shown in Figure 6, all substrate anionic heteroatoms are at a distance between 3.5 and 4.8 Å from a common point C^+. The corresponding main distances and angle are reported in Table 4.

Using the same procedure, calculations were made to see whether it was possible that the 12 substrates, *in their protonated form*, could establish a hydrogen bond with a CYP2C9 acceptor site (see Results).

RESULTS

Oxidation of Tienilic Acid Derivatives and Suprofen by Yeast-Expressed Human CYP2C9. (A) Nature and Synthesis of the Compounds Studied. Tienilic acid is a very good substrate of CYP2C9 with K_m and k_{cat} values for its 5-hydroxylation of 6 μM and 2.4 min^{-1} (Table 1). In order to evaluate the role of the COOH function of tienilic acid in its recognition by CYP2C9, several derivatives having a $COOCH_3$, $CONH_2$, CH_2OH, or CHN_4 (tetrazole) function instead of the COOH group have been prepared. Compounds with the OCH_2COOH group of tienilic acid replaced by OH or OCH_3 groups have also been studied. The synthesis of these compounds from the previously reported phenol precursor **5** is depicted in Figure 2 and described in Materials and Methods. The structure of the new compounds has been established from ^1H NMR and mass spectroscopy, and elemental analysis (see Materials and Methods). Two other 2-aroylthiophenes having an aryl group without the two chlorine substituents of tienilic acid, and either a *p*-OCH_2-COOH (**8**) or a *p*-Cl (**7**) substituent (Table 2), were also studied.

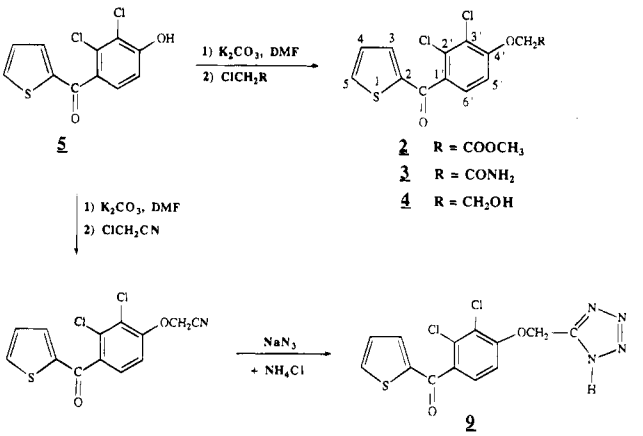


FIGURE 2: Structures and synthesis of several new 2-aroylthiophenes related to tienilic acid.

(B) Assay Used for Studying the P4502C9-Dependent 5-Hydroxylation of Tienilic Acid Derivatives. All these tienilic acid derivatives exhibit a 2-aroylthiophene structure. It has been reported that many 2-aroylthiophenes are hydroxylated at position 5 of their thiophene ring by cytochromes P450 and that the corresponding 5-hydroxy-2-aroylthiophene metabolites can be easily detected by UV−visible spectroscopy at wavelengths around 385 nm (Neau et al., 1990). This spectral feature is due to the existence of 5-hydroxy-2-aroylthiophenes in a highly conjugated anionic

Table 2: 5-Hydroxylation of 2-Aroylthiophenes Related to Tienilic Acid by Yeast-Expressed CYP2C9[a]

compound	R_1	R_2	R_3	specific activity[b]	K_m (μM)	k_{cat} (min^{-1})
1	OCH_2COOH	Cl	Cl	1.9 ± 0.1	6 ± 0.2	2.4 ● 0.1
2	OCH_2COOCH_3[c]	Cl	Cl	<0.02[d]		
3	OCH_2CONH_2	Cl	Cl	<0.02[d]		
4	OCH_2CH_2OH	Cl	Cl	<0.02[d]		
5	OH	Cl	Cl	0.61 ± 0.1	130 ± 12	1.3 ± 0.05
6	OCH_3	Cl	Cl	<0.02[d]		
7	Cl	H	H	<0.02[d]		
8	OCH_2COOH	H	H	0.32 ± 0.06	77 ± 16	0.77 ± 0.1
9	OCH_2CHN_4	Cl	Cl	0.16 ± 0.05	12 ± 1	0.23 ± 0.05
suprofen	$CH(CH_3)COOH$	H	H	0.62 ± 0.08	45 ± 5	0.92 ± 0.1

[a] R_1, R_2, and R_3 refer to the substituents at positions *para*, *meta*, and *ortho* of the aryl ring of 2-aroylthiophenes. [b] Initial rates in nmol of 5-hydroxylated product·(nmol of P450)$^{-1}$·min^{-1} measured after a 10 min incubation of yeast microsomes (0.2 μM P450) in 0.1 M phosphate buffer, pH 7.4, in the presence of 100 μM substrate and a NADPH-generating system, assuming an ϵ at 390 nm identical for all 5-hydroxy-2-aroylthiophene metabolites (see text). [c] Experiments performed in the presence of 10 μM paraoxon; we checked that 10 μM paraoxon did not inhibit the 5-hydroxylation of tienilic acid under identical conditions. [d] Under the detection limit of the assay, it was checked that no activity results even when 500 μM substrate was used. Values are the mean ± SD from three to five independent experiments.

Scheme 1: Major Forms under Which 5-Hydroxy-2-aroylthiophenes Exist at Physiological pH

form at pH 7.4, because of their low pK_a (~3) (Neau et al., 1990) (Scheme 1). This was the basis of the assay used for following the 5-hydroxylation of tienilic acid, suprofen, and compounds **6** and **7** by rat liver microsomes (Neau et al., 1990), as well as the 5-hydroxylation of tienilic acid by CYP2C9 produced in yeast (Lopez-Garcia et al., 1993). It is noteworthy that the λ_{max} and ϵ of the 5-hydroxy-2-aroylthiophene metabolites of compounds **1**, **6**, **7**, and suprofen are very similar (λ_{max} between 382 and 386 nm and ϵ between 27 and 30 mM^{-1}·cm^{-1}) (Neau et al., 1990). This indicates that the characteristics of the UV−vis spectrum of 5-hydroxy-2-aroylthiophenes are not greatly affected by a change in the nature of the substituent of the aryl ring, at least for the substituents used in this study (Cl, *O*-alkyl, and alkyl). Therefore, the 5-hydroxylation of compounds mentioned in Table 2 can be followed by the technique described for tienilic acid and should lead to 5-hydroxy-2-aroylthiophene metabolites with similar UV−vis features.

(C) Comparison of the Ability of Tienilic Acid Derivatives To Act as CYP2C9 Substrates. Optimal conditions for the 5-hydroxylation of tienilic acid by CYP2C9 produced in *S. cerevisiae* D12 transformed with the pAAH5/MP-4 cDNA expression plasmid (Brian et al., 1989b) were reported previously (Lopez-Garcia et al., 1993, 1994). Under those conditions, the methyl ester and amide of tienilic acid, **2** and **3**, failed to undergo any 5-hydroxylation of their thiophene ring by microsomes from yeast expressing CYP2C9 (Table 2), as shown by the lack of appearance of any significant absorbance around 385 nm even after a 20 min incubation. In fact, in the case of tienilic acid methyl ester (**2**), a weak 5-hydroxylation activity could be detected (six times weaker than that observed with tienilic acid under identical conditions; data not shown). However, this weak activity is most likely due to a microsomal esterase-dependent hydrolysis of **2** followed by a CYP2C9-dependent hydroxylation of tienilic acid, as it is completely suppressed in the presence of 10 μM paraoxon, a well-known esterase inhibitor (Castle, 1988) (Table 2). Proper controls showed that paraoxon had no effect on the microsomal 5-hydroxylation of tienilic acid under identical conditions (data not shown).

Alcohol **4**, which formally derives from the reduction of the carboxylic acid function of tienilic acid, also failed to undergo any 5-hydroxylation of its thiophene ring after a 20 min incubation period with yeast microsomes. An identical result was obtained with 2-aroylthiophenes **6** and **7** that bear an OCH_3 or Cl substituent instead of the OCH_2-COOH function of tienilic acid. Conversely, compound **5** which contains an OH aryl substituent was oxidized by yeast microsomes expressing CYP2C9, giving rise to a 387 nm-absorbing metabolite. The K_m value for this reaction of 130 ± 12 μM (Figure 3) is much higher than the K_m found for tienilic acid, but the k_{cat} of 1.3 ± 0.05 min^{-1} is only slightly smaller than that found for tienilic acid (Table 2). Compound **8**, a carboxylic acid closely related to tienilic acid but without chlorine substituents on the aryl ring, also underwent a 5-hydroxylation of its thiophene ring, giving rise to an absorbance maximum at 384 nm. It is a poorer substrate than tienilic acid for CYP2C9 with a 13-fold higher K_m and 3-fold lower k_{cat} (Table 2 and Figure 3).

The aforementioned results strongly suggested that the COOH function of tienilic acid played a key role in the recognition and 5-hydroxylation of this drug by CYP2C9. Its replacement by a hydrogen atom (compound **6**) or by other polar functions such as $COOCH_3$, $CONH_2$, or CH_2OH (compounds **2**, **3**, and **4**) completely abolished the 5-hydroxylation of the corresponding substrate by CYP2C9. However, compound **5**, which bears an acidic phenol with a pK_a of 6.3 (see Materials and Methods) and should mainly exist in an anionic form at pH 7.4, was found to be 5-hydroxylated by CYP2C9, as is tienilic acid and acid **8**. In order to further confirm the importance of an anionic site in the *para* position of the aryl group of 2-aroylthiophenes for their recognition and 5-hydroxylation by CYP2C9, we prepared compound **9** in which the COOH group of tienilic acid was replaced by a tetrazole function (Figure 2). Such tetrazoles generally exhibit pK_a values similar to those of the corresponding carboxylic acids. Accordingly, the pK_a of **9** was found to be 3.8 (see Materials and Methods), a value not far from that of tienilic acid. Incubation of 100 μM tetrazole **9** with microsomes from yeast expressing CYP2C9 and 1 mM NADPH led to the appearance of a 390 nm-absorbing metabolite. As in the case of the other compounds leading to such metabolites, its formation only

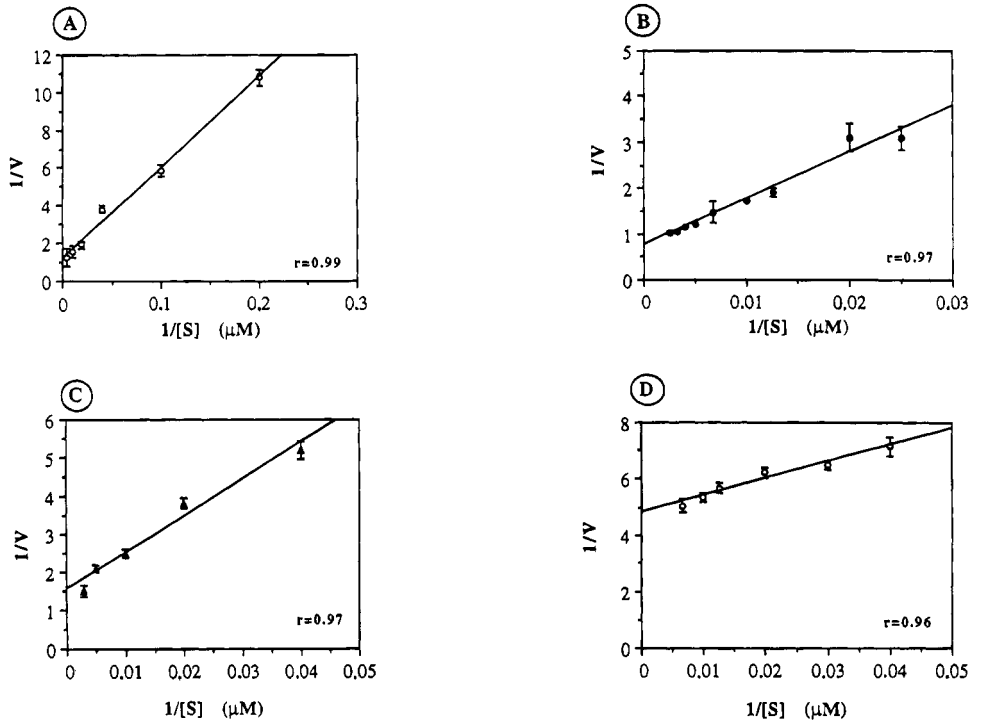


FIGURE 3: Kinetics of 5-hydroxylation of the thiophene ring of suprofen (A) and compounds **5** (B), **8** (C), and **9** (D) by pAAH5/P450 2C9 yeast microsomes. Conditions were identical to those used for tienilic acid 5-hydroxylation (Lopez-Garcia et al., 1993). V = initial rate of formation of 5-hydroxylated product in nmol·(nmol of P450)$^{-1}$·min^{-1}; S = substrate. Data are means ± SD from three independent experiments.

occurred in the presence of NADPH and O_2, did not occur with microsomes of control yeast (not expressing CYP2C9), and was almost completely inhibited in the presence of 50 μM sulfaphenazole, a known inhibitor of CYP2C9 (Miners et al., 1988; Brian et al., 1989a) (data not shown). Kinetic experiments (Figure 3) showed that compound **9** was a relatively good substrate for CYP2C9 with a K_m of 12 ± 1 μM, similar to that of tienilic acid, but a k_{cat} of 0.23 ± 0.05 min^{-1}, much lower than that of tienilic acid (Table 2).

Suprofen, a nonsteroidal antiinflammatory agent, is a 2-aroylthiophene compound comparable to tienilic acid as its contains a carboxylic acid group whose position relative to the thiophene ring is similar to that found in tienilic acid (Table 2). It has been reported that suprofen undergoes a 5-hydroxylation of its thiophene ring in the presence of rat liver microsomes (Neau et al., 1990). This oxidation was also found to be catalyzed by microsomes of yeast expressing CYP2C9 (Table 2). However, suprofen exhibited a higher K_m (about 8-fold) and a lower k_{cat} (3-fold) than tienilic acid (Figure 3 and Table 2).

The results of Table 2 clearly show that, for all the 2-aroylthiophenes studied, only those bearing a negative charge (at pH 7.4) on a *para* substituent of the aryl ring (as in tienilic acid) are 5-hydroxylated on their thiophene ring by CYP2C9. However, these results do not allow us to exclude the possibility that the 2-aroylthiophenes which are not hydroxylated on their thiophene ring might be hydroxylated at another site by CYP2C9. The following experiments have been performed to study this possibility.

Inhibition of the CYP2C9-Dependent Hydroxylation of Tienilic Acid by Various Compounds with Respect to Their Containing an Acidic Moiety. The ability of compounds **3**, **4**, **6**, and **7**, which failed to undergo any CYP2C9-dependent 5-hydroxylation of their thiophene ring, to be recognized by CYP2C9 was addressed by studying their ability to inhibit

Table 3: Inhibition of CYP2C9-Dependent Hydroxylation of Tienilic Acid by 2-Aroylthiophene Derivatives, CYP2C9 Substrates, and Some Nonsteroidal Antiinflammatory Drugs

	IC_{50} (μM)[a]	K_i (μM)
3	>1000[b]	
4	>1000[b]	
6	>1000[b]	
7	>1000[b]	
phenytoin	81	10 ± 1
warfarin	28	3.5 ± 0.5
diclofenac	13	2 ± 0.2
ibuprofen	390	50 ± 6
ketoprofen	308	42 ± 4
naproxen	>1000[b]	

[a] IC_{50} values were measured by using increasing concentrations of potential inhibitors added to the assay incubation mixture including pAAH5/P450 2C9 microsomes (0.2 μM P450) in phosphate buffer, pH 7.4, 50 μM tienilic acid, and a NADPH-generating system. [b] No significant inhibitory effects even with 1000 μM compound; for those compounds, the lack of any inhibitory effect was also observed when a [compound]/[tienilic acid] ratio of 100 was used ([tienilic acid] = 10 μM). Values are the mean ± SD from three to four experiments.

CYP2C9-dependent hydroxylation of tienilic acid by yeast microsomes. As shown in Table 3, none of them led to any inhibitory effect even at 1000 μM. These results indicate that the nonacidic compounds **3**, **4**, **6**, and **7** not only are not hydroxylated at position 5 of their thiophene ring by CYP2C9 but also are not recognized by this cytochrome.

Conversely, known substrates of CYP2C9, (*S*)-warfarin and phenytoin, clearly inhibited the hydroxylation of tienilic acid in a competitive manner (Figure 4) with K_i values of 3.5 ± 0.5 and 10 ± 1 μM (Table 3). The nonsteroidal antiinflammatory drugs which contain a carboxylic acid function, ibuprofen, ketoprofen, and diclofenac, were also found to be good competitive inhibitors of tienilic acid hydroxylation by yeast microsomes, with K_i values of 50 ±

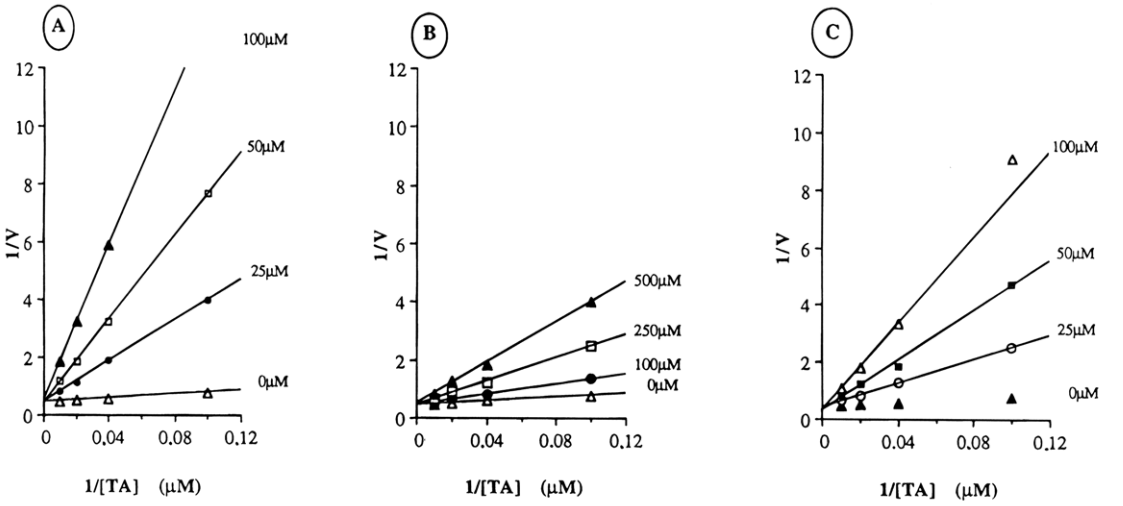


FIGURE 4: Representative Lineweaver–Burk plots for the inhibition of pAAH5/P450 2C9 yeast microsome-dependent 5-hydroxylation of tienilic acid by diclofenac (A), ibuprofen (B), and (*S*)-warfarin (C). Panels show the plot of the reciprocal of initial 5-hydroxytienilic acid formation rates (determined under standard conditions: 10-min incubation, 0.2 μM P450) vs 1/[TA] (TA = tienilic acid) in the presence of various inhibitor concentrations.

6, 42 ± 4, and 2 ± 0.2 μM (Figure 4, Table 3). Another NSAID, naproxen, which is also an arylpropionic acid, did not lead to any inhibitory effect even for a naproxen:tienilic acid ratio of 100.

A Predictive Model for Substrates of CYP2C9 Derived from Molecular Modeling Techniques. Most substrates reported so far for CYP2C9, including those described in this paper (Figure 1 and Table 2), contain an acidic hydrogen exhibiting a pK_a between 3.8 and 8.1. Molecular modeling calculations were thus made on 12 substrates of CYP2C9 reported so far (the 10 compounds shown in Figure 1 plus the two tienilic acid derivatives, **5** and **9**, that were found to act as substrates of CYP2C9) in order to have an idea of the substrate binding site of CYP2C9. The model was constructed by considering the relative position of the anionic (or acidic) site and the site of hydroxylation by CYP2C9 (Table 1) in these substrates. Two hypotheses appeared possible *a priori*. The most probable one involves the binding of substrates under their anionic form *via* an interaction of their anionic site with a cationic site of the protein. The second one would involve the binding of substrates under their acid form through a hydrogen bond to a protein site.

In order to test the first hypothesis, two rigid substrates, phenytoin and (*S*)-warfarin, for which X-ray structures have been published (Camernan & Camernan, 1971; Valente et al., 1975, respectively), were superimposed with respect to their major hydroxylation site (see Table 1) and the aromatic ring bearing this hydroxylation site. The conformations from the X-ray structures were used initially. Thus (*S*)-warfarin existed in a very rigid tricyclic conformation exhibiting a cyclic hemiketal function (Valente et al., 1975) (Chart 1). Figure 5 shows that the N^- and O^- atoms of phenytoin and warfarin are then separated by less than 2 Å. Tienilic acid was then overlayed onto phenytoin and warfarin in the same manner; its conformation was taken from X-ray crystallographic data (Carpy et al., 1980). With such an arrangement of the three substrates, it was easy to find a focal point [a putative cationic site on the protein (C^+ in Figure 5)] approximately at 4 Å from each of their anionic sites, this distance being compatible with a favorable ionic interaction (Islam et al., 1991). The N^- site of phenytoin was at the

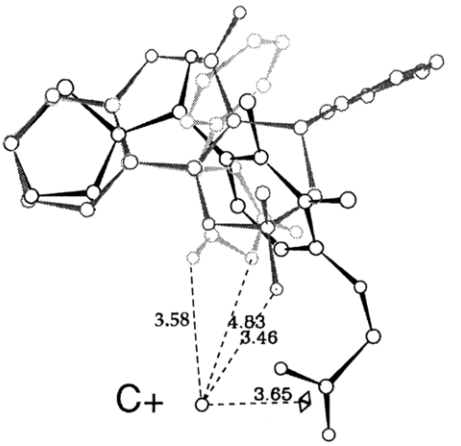
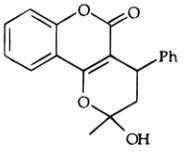


FIGURE 5: Superposition of the hydroxylation sites and hydroxylated aromatic rings of warfarin, phenytoin, and tienilic acid. Possible interaction of their anionic sites with a cationic site of CYP2C9 (C^+) is shown. The conformations of the three substrates are drawn from their X-ray crystallographic structure.

Chart 1: Tricyclic Hemiketal Conformation Found in the X-ray Crystallographic Structure of (*S*)-Warfarin (Valente et al., 1975)



longest distance (4.8 Å); however, one of its carbonyl oxygen atoms which also bears part of the negative charge was closer (3.6 Å) (Table 4). Next, the hydroxylation sites of the 12 substrates shown in Table 4 were superimposed, and these substrates were arranged in order to have their anionic atom at a distance between 2.5 and 4.5 Å from a common putative cationic site on the protein (as C^+ in Figure 5). It was possible to find such an arrangement (Figure 6) by using the conformations given by the X-ray crystallographic structures of diclofenac (Reck et al., 1988a), ibuprofen (Freer, 1993), phenytoin, piroxicam (Reck et al., 1988b), tolbutamide (Donaldson et al., 1981), (*S*)-warfarin, and tienilic acid and the minimum energy conformations calculated for suprofen, tenoxicam, and 58C80 with the Biosym cvff force field. In

Table 4: Main Distances and Angles Found from the Modeling of the Substrate Binding Site of CYP2C9[a]

substrate	conformation used[b]	distances and angle		
		HyA^- (Å)	A^-C^+ (Å)	HyA^-C^+ (deg)
diclofenac	X-ray	7.37	4.48	85
ibuprofen	X-ray	7.9	3.73	82
phenytoin	X-ray	5.93 (5.45)	4.83 (3.58)	100 (117)
piroxicam	X-ray	7.75 (5.28)	3.57 (3.86)	85 (129)
suprofen	$\Delta E = 0$	9.88	4.4	56
tenoxicam	$\Delta E = 0$	7.75 (5.21)	3.57 (3.86)	85 (129)
tolbutamide	X-ray	6.68 (6.5)	3.78 (4.9)	101 (85)
(*S*)-warfarin	X-ray	7.15	3.46	96
58C80	$\Delta E = 0$	6.86	3.7	98
1	X-ray	8.71	3.65	70
5	$\Delta E = 8$	8.56	4.2	70
9	$\Delta E = 4$	9.56 (9.84)	4.33 (3.57)	60 (58)
mean ± SD		7.84 ± 1.58	3.97 ± 0.43	82.3 ± 14.7

[a] This model is shown in Figure 6; its construction is explained in the text. [b] Hy, A^-, and C^+ are the hydroxylation and anionic sites of substrates and the positively charged atom of an amino acid residue of CYP2C9. Conformations used for constructing the model were either drawn from X-ray crystallographic structure data (X-ray in column 2) or deduced from energy minimization calculations; $\Delta E = 0$ corresponds to minimum energy conformations and $\Delta E = x$ to conformations slightly higher in energy (by x kcal) than the minimum energy conformation. All values that are outside of parentheses correspond to the substrate A heteroatom originally bearing an hydrogen atom in the acidic form (in fact, in carboxylic acid containing substrates, A is the middle of the O---O segment, and in **9**, A is the closest N atom of the tetrazole ring). Values in parentheses correspond to the other substrate heteroatom on which the negative charge may be delocalized (O for phenytoin, carbonyl O for piroxicam and tenoxicam, carbonyl O for tolbutamide). In **9**, values in parentheses correspond to A in the center of the tetrazole ring.

the case of compounds **5** and **9**, conformations that were higher in energy by 8 and 4 kcal, respectively, than the corresponding minimum energy conformations were used. Table 4 shows that, for the 12 substrates, the distance A^-C^+ between their anionic site and the possible CYP2C9 cationic site lies between 3.46 Å [for (*S*)-warfarin] and 4.8 Å for phenytoin, with a mean value around 4 Å. This situation is analogous, with an inversion of the charges, to that proposed for the CYP2D6 active site (Wolff et al., 1985; Meyer et al., 1986; Koymans et al., 1992; Strobl et al., 1993) in which the substrate cationic sites are at a distance between 2.5 and 4.5 Å from an anionic residue on the protein (Islam et al., 1991). In fact, most of the deprotonated substrates of CYP2C9 have their anionic charge delocalized on two or three heteroatoms. This is naturally the case of all substrates bearing a COO^- function, where the negative charge should be equally localized on the two oxygen atoms. The negative charge of deprotonated phenytoin is localized not only on its deprotonated nitrogen but also on the two oxygen atoms of adjacent CO groups. In a similar manner, the negative charge of deprotonated tolbutamide is localized not only on its deprotonated nitrogen but also, at least partly, on the three oxygen atoms of adjacent SO_2 and CO groups. In the case of tenoxicam, piroxicam, and 58C80, the negative charge could be delocalized between their deprotonated OH group and the oxygen atom of the conjugated CO group. In tetrazole **9**, the negative charge is equally delocalized among the four nitrogen atoms of the tetrazole cycle. Conversely, in phenol **5** and the tricyclic conformation of warfarin, the negative charge is mainly localized on a single oxygen atom. In fact, in the model of Figure 6, we checked that all the distances between the protein cationic site (blue point in Figure 6 and C^+ in Figure 5) and the negatively charged atoms of deprotonated substrates remained between 3.5 and 4.5 Å, whatever the nature of the negatively charged heteroatom (except for the CO oxygen atom of tolbutamide and for the N atom of phenytoin for which a distance around 4.8 Å was found). The HyA^- distance between the hydroxylation site Hy and the negatively charged heteroatom A^- of the substrates varies between 5.93 (phenytoin) and 9.88 Å (suprofen) with a mean value ± SD of 7.8 ± 1.6 Å. In fact, if one omits suprofen and **9** which exhibit particularly long HyA^- distances, the other 10 substrates exhibit a mean distance of 7.5 ± 0.8 Å. In the model of Figure 6 the $\angle HyA^-C^+$ angle has a value of 82 ± 15°, with extreme values of 56° for suprofen and 101° for tolbutamide.

Similar superpositions of substrates and calculations were carried out in order to test the hypothesis of the binding of substrates under their acid form through a hydrogen bond to a protein site. Seven CYP2C9 substrates, phenytoin, (*S*)-warfarin, tienilic acid, tenoxicam, tolbutamide, suprofen, and **5**, were overlayed as before. Their final positions were optimized in order to allow a distance ~2 Å (Böhm, 1992) between their acidic hydrogen and a putative amino acid heteroatom from the protein which would form a hydrogen bond with the substrate. Final allowed substrate conformations used in the model were kept within a 10 kcal·mol^{-1} range above the energy minimum. In the best system, several distances between the substrate acidic hydrogen and the heteroatom from the protein were compatible with a hydrogen bond (between 1.7 and 2.6 Å) (data not shown). However, for two substrates, suprofen and **5**, the corresponding distances were too long for an hydrogen bond (3.9 and 3.8 Å, respectively). Furthermore, the $\angle AHP$ angle (A being the substrate atom bearing the acidic hydrogen and P the atom from the protein involved in the hydrogen bond) found for several substrates was too small for a hydrogen bond (82, 90, and 100 for suprofen, **5**, and warfarin).

DISCUSSION

Only a few substrates of CYP2C9 have been reported in the literature (see, for instance, recent reviews: Smith & Jones, 1992; Goldstein & de Morais, 1994b; Guengerich, 1995). It was established without ambiguity for phenytoin, tolbutamide, (*S*)-warfarin, and tienilic acid by using CYP2C9 expressed in heterologous systems, as well as for 58C80 by using purified CYP2C9. Other substrates have been proposed on the basis of experiments performed on human liver microsomes and compounds believed to be either selective substrates or selective inhibitors of CYP2C9. This was the case of NSAIDs such as diclofenac, ibuprofen, piroxicam, tenoxicam, naproxen, and mefenamic acid (Bonnabry et al., 1993) and of tetrahydrocannabinol (Bornheim et al., 1992). However, several P450 isoforms including several members of the CYP2C subfamily are present in human liver microsomes. Thus, it will be difficult to definitely conclude that a compound is a substrate for CYP2C9 from experiments using such microsomes and known CYP2C9 substrates and inhibitors until the complete selectivity of these substrates and inhibitors toward CYP2C9 is demonstrated. Results of Table 3 clearly confirm that diclofenac and ibuprofen are well recognized by CYP2C9 with a good affinity, contrary to naproxen which fails to inhibit CYP2C9 and which should be a very poor substrate of this isozyme. In this work, we also show (Table 2) that another NSAID, suprofen, and three

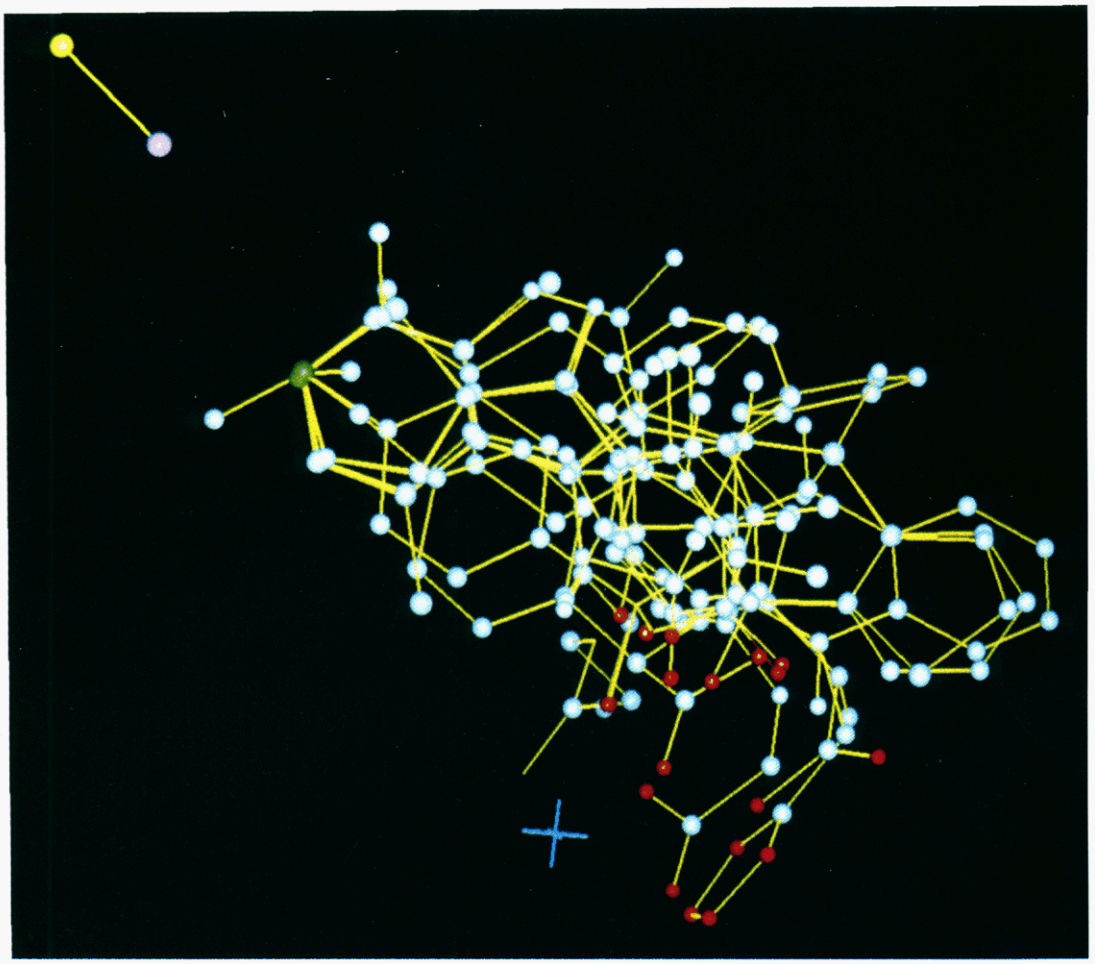


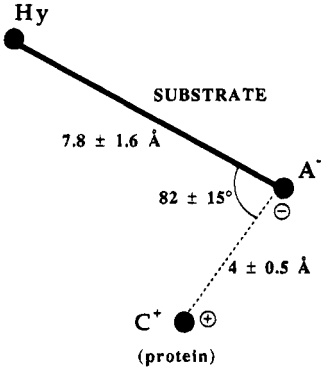
FIGURE 6: Active site model of CYP2C9 when 12 substrates are fitted onto the template molecules warfarin, phenytoin, and tienilic acid and when looking for a common point (in blue) located at a distance between 2.5 and 4.5 Å of the substrate anionic sites (in red). The hydroxylation sites of all substrates are superimposed (in green). The blue point would represent a cationic amino acid residue on the distal side of the CYP2C9 active site (for all detailed data, see Table 4). The CYP2C9 iron (pink spot) and the proximal sulfur ligand (yellow spot) are only indicated to recall that the iron should be at a distance ~4.2 Å from the hydroxylation site (by analogy with that found in P450 cam; Poulos et al., 1987). The relative positions of Fe and proximal S from the rest of the substrate molecules are not known.

other 2-aroylthiophenes, **5**, **8**, and **9**, are new substrates of CYP2C9. Therefore, the following 10 compounds that are established as CYP2C9 substrates, namely, phenytoin, tolbutamide, (*S*)-warfarin, 58C80, tienilic acid, suprofen, ibuprofen, diclofenac, **5**, and **9**, were mainly used to better understand the substrate binding site of CYP2C9. The results obtained with these 10 compounds were then applied to two other drugs that have been proposed to be CYP2C9 substrates on the basis of results of experiments performed with human liver microsomes, piroxicam and tenoxicam (Zhao et al., 1992), taking into account their structural analogy with the 10 substrates mentioned above.

These 12 compounds are protic acids with a pK_a below 7 except for phenytoin which exhibits a pK_a of 8.1, only slightly higher than the pH of 7.4 used for incubation experiments. Moreover, results of Table 2 show, from 10 compounds related to tienilic acid, that all exhibit a 2-aroylthiophene framework, and various charged or uncharged chemical functions in the *para* position of the aryl ring, only those existing as anions at pH 7.4, act as substrates of the CYP2C9-catalyzed 5-hydroxylation of their thiophene ring. Whatever the nature of the chemical function leading to an anion at pH 7.4 (phenol as in **5**, tetrazole as in **9**, or carboxylic acid as in **1** and **8**), the presence of this anion on the aryl ring of the substrate is a key factor for its recognition by CYP2C9. On the contrary, closely related 2-aroylthiophenes bearing various uncharged polar functions such as esters, amides, alcohols, or ethers (**2**, **3**, **4**, and **6**), which have the potential to form hydrogen bonds with CYP2C9, do not undergo a 5-hydroxylation of their thiophene ring (Table 2) and fail to act as inhibitors of CYP2C9 (Table 3). Furthermore, preliminary results using purified CYP2C9 show that tienilic acid and suprofen interact well with CYP2C9, giving rise to type I UV−vis difference spectra characterized by peaks around 390 nm as expected for the formation of a P450 2C9−substrate complex (with respective K_s values of 6 and 50 μM not far from the K_m values found for these compounds, Table 2), whereas compounds **3**, **4**, **6**, and **7** failed to give any difference spectrum (A. Mancy, S. Poli, and D. Mansuy, to be published).

These results clearly establish the key role of the presence of an anionic site in substrates for their recognition by CYP2C9 and suggest an ionic interaction of this site with a cationic amino acid residue from the distal side of the CYP2C9 protein. This suggestion was found to be in agreement with the results of molecular modeling studies, showing that the superposition of 12 substrates of CYP2C9 with respect to their hydroxylation sites leads to an arrangement of these molecules in which all of their anionic sites are at a distance around 4 Å from a common point in space (the putative cationic site of the protein) (Figure 6 and Table 4). A predictive model for substrates in the active site of

Chart 2: Schematic View of a Possible Substrate Binding Site of CYP2C9[a]



[a] From data of Figure 6 and Table 4. HyA^- is the substrate, Hy being the site of hydroxylation by CYP2C9 and A^- the anionic site. C^+ is a putative cationic amino acid residue of the protein.

CYP2C9 drawn from these results is schematically shown in Chart 2. It involves three points: Hy, the substrate atom hydroxylated by CYP2C9, whose distance to the P450 iron would be around 4.2 Å (Poulos et al., 1987); A^-, the substrate atom bearing the negative charge; and C^+, the cationic amino acid residue from the CYP2C9 protein. In this model, the HyA^- and A^-C^+ distances are 7.8 ± 1.6 and 4 ± 0.4 Å, and the $\angle HyA^-C^+$ angle is 82 ± 15°.

The aforementioned molecular modeling data are much less in favor of another possible way of recognition of substrates in their protonated form *via* a hydrogen bond with a protein acceptor (see above). Accordingly, amide **3** and alcool **4**, which should give such a hydrogen bond, are not recognized by CYP2C9 (Tables 2 and 3).

The model outlined in Chart 2 insists that the interaction with C^+ be a major determinant of substrate recognition by CYP2C9. Other structural factors that remain to be determined must be important for this recognition. From the model shown in Figure 6, the presence of an aromatic ring close to Hy (and thus close to the heme) seems to be important, as 9 of the 12 substrates considered undergo a hydroxylation of their aromatic ring. Moreover, the presence of hydrophobic, sometimes bulky, substituents above the HyA^- segment of the HyA^-C^+ triangle (Chart 2) is found in many substrates. Interaction of the protein with such a substituent could play an important role as shown by the much less efficient hydroxylation of **8** (Table 2) which only differs from tienilic acid by two Cl substituents. Conversely, the region to the left of the HyC^+ segment seems to be empty, which suggests that the CYP2C9 active site may accept bulky groups on the right but not on the left of the HyC^+ segment. Naturally, Figure 6 and Chart 2 only provide an initial schematic model of the substrate binding site of CYP2C9. Many experiments and more sophisticated calculations are necessary to confirm and to refine this model. However, whatever its precise structure might be, the substrate binding site of CYP2C9 appears to involve a cationic site favoring the recognition of negatively charged substrates. This explains why CYP2C9 is so often involved in the metabolic oxidation of nonsteroidal antiinflammatory drugs (Leemann et al., 1993b) that very often contain a carboxylic acid or another acidic function. This also explains a very recent result indicating that arachidonic acid is a substrate of CYP2C9 (Daikh et al., 1994).

ACKNOWLEDGMENT

We thank Prof. F. Guengerich and Prof. P. Beaune for providing us with yeast expressing CYP2C9, O. Courtin for helpful discussions, N. N. Quang and P. Pouzet for the synthesis of some 2-aroylthiophenes, and Anphar-Rolland Laboratories (Chilly-Mazarin, France) for providing us with tienilic acid and 5-hydroxytienilic acid.

REFERENCES

Alexander, R. R., & Griffiths, J. M. (1993) in *Basic Biochemicals Methods*, pp 7–16, Wiley-Liss, New York.
Aoyama, T., Yamano, S., Guzelian, P. S., Gelboin, H. V., & Gonzalez, F. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 4790–4793.
Böhm, H. J. (1992) *J. Comput.-Aided Mol. Des. 6,* 61–78.
Bonnabry, P., Leemann, T., & Dayer, P. (1993) *Clin. Pharmacol. Ther. 55,* 139.
Borheim, L. M., Lasker, J. M., & Raucy, J. L. (1992) *Drug Metab. Dispos. 20,* 241–246.
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.
Camerman, N., & Camerman, A. (1971) *Acta Crystallogr. B27,* 2205–2211.
Carpy, A., Goursolle, M., & Leger, J. M. (1980) *Acta Crystallogr. B36,* 1706–1708.
Castle, M. C. (1988) *Arch. Int. Pharmacodyn. 295,* 7–16.
Daikh, B. E., Lasker, J. M., Raucy, J. L., & Koop, D. R. (1994) *J. Pharmacol. Exp. Ther. 271,* 1427–1433.
Doecke, C. J., Veronese, M. E., Pond, S. M., Miners, J. O., Birkett, D. J., Sansom, L. N., & McManus, M. E. (1991) *Br. J. Clin. Pharmacol. 31,* 124–130.
Donaldson, J. D., Leary, J. R., Ross, S. D., Thomas, M. J. K., & Smith, C. H. (1981) *Acta Crystallogr. B37,* 2245–2248.
Freer, A. A. (1993) *Acta Crystallogr. C49,* 1378–1380.
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., & de Morais, S. M. F. (1994b) *Pharmacogenetics 4,* 285–299.
Goldstein, J. A., Faletto, M. B., Romkes-Sparks, M., Sullivan, T., Kitareewan, S., Raucy, J. L., Lasker, J. M., & Ghanayem, B. I. (1994a) *Biochemistry 33,* 1743–1752.
Gonzalez, F. J. (1989) *Pharmacol. Rev. 40,* 243–288.
Guengerich, F. P. (1995) in *Cytochrome P450, Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., Ed.) 2nd ed., Plenum Press, New York (in press).
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.
Herbert, M., & Pichat, L. (1976) *J. Labelled Compd. Radiopharm. 12,* 437–453.
Islam, S. A., Wolf, C. R., Lennard, M. S., & Sternberg, M. J. E. (1991) *Carcinogenesis 12,* 2211–2219.
Jones, B. C., Hawksworth, G., Horne, V., Newlands, A., Tute, M., & Smith, D. A. (1994) in *Cytochrome P450* (Lechner, M. C., Ed.) pp 523–525, John Libbey Eurotext Ltd., Montrouge, France.
Kadaba, P. K. (1973) *Synthesis,* 71–84.
Koymans, L., Vermeulen, N. P. E., Van Acker, S. A. B. E., Te Koppele, J. M., Heykants, J. J. P., Lavrijsen, K., Meuldermans, W., & Donné-Op den Kelder, M. (1992) *Chem. Res. Toxicol. 5,* 211–219.
Leemann, T., Transon, C., & Dayer, P. (1993a) *Life Sci. 52,* 29–34.
Leemann, T. D., Transon, C., Bonnabry, P., & Dayer, P. (1993b) *Drugs Exp. Clin. Res. 19,* 189–195.
Leemann, T., Bonnabry, P., & Dayer, P. (1994) *Proceedings of the 14th European Workshop on Drug Metabolism,* p 140, Paris.
Levron, J. C., Le Fur, J. M., Adnot, P., & Dormard, Y. (1977) *Eur. J. Drug Metab. Pharmacokinet. 3,* 107–120.

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.
Lopez Garcia, M. P., Dansette, P. M., & Mansuy, D. (1994) *Biochemistry 33,* 166−175.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265−275.
Mansuy, D., Dansette, P., Foures, C., Jaouen, M., Moinet, G., & Bayer, N. (1984) *Biochem. Pharmacol. 33,* 1429−1435.
Meyer, U. A., Gut, J., Kronbach, T., Skoda, C., Meier, U. T., & Catin, T. (1986) *Xenobiotica 16,* 449−464.
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.
Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., & Nebert, D. W. (1993) *DNA 12,* 1−51.
Newlands, A. J., Smith, D. A., Jones, B. C., & Hawksworth, G. M. (1992) *Br. J. Clin. Pharmacol. 34,* 152P.
Ohgiya, S., Komori, M., Fujitani, T., Miura, T., Shinriki, N., & Kamataki, T. (1989) *Biochem. Int. 13,* 429−438.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239,* 2370−2378.
Opong-Mensah, K., Woller, T. W., Obaseki, A. O., & Porter, W. R. (1984) *J. Pharm. Biomed. Anal. 2,* 381−394.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195,* 687−700.
Reck, G., Faust, G., & Dietz, G. (1988a) *Pharmazie 43,* 771−774.
Reck, G., Dietz, G., Laban, G., Guenther, W., Bannier, G., & Hoehne, E. (1988b) *Pharmazie 43,* 477−481.
Relling, M. V., Aoyama, T., Gonzalez, F. J., & Meyer, U. A. (1990) *J. Pharmacol. Exp. Ther. 252,* 442−447.
Rettie, A. E., Korzekwa, K. R., Kunze, K. L., Lawrence, R. F., Eddy, A. C., Aoyama, T., Gelboin, H. V., Gonzalez, F. J., & Trager, W. F. (1992) *Chem. Res. Toxicol. 5,* 54−59.
Rettie, A. E., Wienkers, L. C., Gonzalez, F. J., Trager, W. F., & Korzekwa, K. R. (1994) *Pharmacogenetics 4,* 39−42.
Romkes, M., Faletto, M. B., Blaisdell, J. A., Raucy, J. L., & Goldstein, J. A. (1991) *Biochemistry 30,* 3247−3255.
Rosenberg, L. S., & Jackson, J. L. (1989) *Drug Dev. Ind. Pharm. 15,* 373−386.
Sandhu, P., Baba, T., & Guengerich, F. P. (1993) *Arch. Biochem. Biophys. 306,* 443−450.
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.
Strobl, G. R., von Kruedener, S., Stöckigt, J., Guengerich, F. P., & Wolff, T. (1993) *J. Med. Chem. 36,* 1136−1145.
Umbenhauer, D. R., Martin, M. V., Lloyd, R. S., & Guengerich, F. P. (1987) *Biochemistry 26,* 1094−1099.
Valente, E. J., Trager, W. F., & Jensen, L. H. (1975) *Acta Crystallogr. B31,* 954−960.
Veronese, M. E., Mackenzie, P. I., Doecke, C. J., McManus, J. O., Miners, J. O. & Birkett, D. J. (1991) *Biochem. Biophys. Res. Commun. 175,* 1112−1118.
Veronese, M. E., Doecke, C. J., Mackenzie, P. I., McManus, M. E., Miners, J. O., Rees, D. L. P., Gasser, R., Meyer, U. A., & Birkett, D. J. (1993) *Biochem. J. 289,* 533−538.
Weaver, R. J., Dickins, M., & Burke, M. D. (1993) *Biochem. Pharmacol. 46,* 1183−1197.
Wolff, T., Distlerath, L. M., Worthington, M. T., Groopman, J. D., Hammons, G. J., Kadlubar, F. F., Prough, R. A., Martin, M. V., & Guengerich, F. P. (1985) *Cancer Res. 45,* 2116−2122.
Yasumori, T., Murayama, N., Yamazoe, Y., Abe, A., Nogi, Y., Fukasawa, T., & Kato, R. (1989) *Mol. Pharmacol. 35,* 443−449.
Zhao, J., Leemann, T., & Dayer, P. (1992) *Life Sci. 51,* 575−581.

BI950622A