Diclofenac and Its Derivatives As Tools for Studying Human Cytochromes P450 Active Sites: Particular Efficiency and Regioselectivity of P450 2Cs

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ABSTRACT: A comparison of the oxidations of diclofenac with microsomes of yeasts expressing various human liver cytochromes P450 showed that P450 2C9 regioselectively led to 4′-hydroxy diclofenac (4′-OHD) whereas P450 3A4 only led to 5-hydroxy diclofenac (5-OHD). P450 2C19, 2C18, and 2C8 led to the simultaneous formation of 4′-OHD and 5-OHD (respective molar ratios of 1.3, 0.37, and 0.17), and P450 1A1, 1A2, 2D6, and 2E1 failed to give any detectable hydroxylated metabolite under identical conditions. P450 2C9 was found to be much more efficient for diclofenac hydroxylation than all the other P450s tested (k_{cat}/K_M of 1.6 min^{-1} μM^{-1} instead of 0.025 for the second more active P450), mainly because of markedly lower K_M values (15 ± 8 instead of values between 170 and 630 μM). Oxidation of diclofenac with chemical model systems of cytochrome P450 based on iron porphyrin catalysts exclusively led to the quinone imine derived from two-electron oxidation of 5-OHD, in an almost quantitative yield. Two derivatives of diclofenac lacking its COO^- function were then synthesized; their oxidation by recombinant human P450 2Cs always led to a major product coming from their 5-hydroxylation. Substrate **2**, which derives from reduction of the COO^- function of diclofenac to the CH_2OH function, was studied in more detail. All the P450s tested (1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, and 3A4) almost exclusively led to its 5-hydroxylation. P450s of the 2C subfamily were found to be the most efficient catalysts for this reaction, with k_{cat}/K_M values between 0.2 and 1.6 min^{-1} μM^{-1}. Oxidation of **2** with an iron porphyrin-based chemical model of cytochrome P450 also led to a product derived from the oxidation of **2** at position 5. These results show that oxidation of diclofenac and its derivative **2**, either with chemical model systems of cytochrome P450 or with recombinant human P450s, generally occurs at position 5. This position, para to the NH group on the more electron-rich aromatic ring of diclofenac derivatives, is thus, as expected, the priviledged site of reaction of electrophilic, oxidant species. The most spectacular exception to this chemoselective 5-oxidation of diclofenac derivatives was found for oxidation of diclofenac itself with P450 2C9 (and P450 2C19 and 2C18 to a lesser extent), which only led to 4′-OHD. A likely explanation for this result is a strict positioning of diclofenac in the P450 2C9 active site, via its COO^- function, to completely orientate its hydroxylation toward position 4′, which is not chemically preferred. P450 2C19, 2C18, and 2C8 would not lead to such a strict positioning as they give mixtures of 4′-OHD and 5-OHD. The above results show that diclofenac derivatives are interesting tools to compare the active site topologies of human P450 2Cs.

The oxidative metabolism of drugs and other xenobiotics in man is in great part under the control of cytochrome P450-dependent monooxygenases. 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, human liver microsomes, and recombinant enzymes in the presence of specific substrates and inhibitors of the various human P450s. P450s of the 3A and 2C subfamilies are the major isozymes in human liver (*1*). Moreover, they are, with CYP 2D6, the P450s most often encountered in the metabolism of pharmaceuticals in humans (*2*).

Diclofenac (Figure 1) is a nonsteroidal antiinflammatory drug that is widely used for the treatment of osteoarthritis and rheumatoid arthritis, ankylosing spondylitis, and acute muscle pain conditions. In vivo studies in man have shown the presence in urine and plasma of various metabolites deriving from the hydroxylation of diclofenac at positions 3′, 4′, and 5 (Figure 1) of its aryl rings (*3*−*5*). In vitro studies using human liver microsomes have shown the major formation of 4′-hydroxy-diclofenac (4′-OHD), which is mainly catalyzed by P450 2C9[1] (*6*, *7*), and the minor

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[1] Abbreviations: P450 or CYP, cytochrome P450; 4′-OHD, 4′-hydroxy diclofenac; 5-OHD, 5-hydroxy diclofenac; s, singlet; d, doublet; t, triplet; m, massif; bs, broad singlet; dd, doublet of doublets; TDCPP, *meso*-tetra(2,6-dichlorophenyl)porphyrin.

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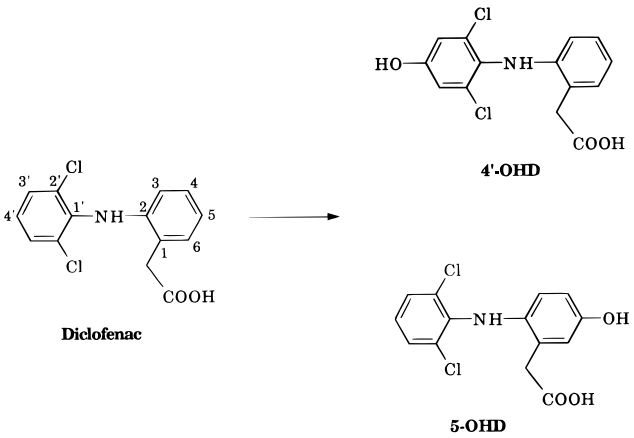


FIGURE 1: Main metabolites derived from oxidation of diclofenac with human liver microsome.

formation of 5-hydroxy diclofenac (5-OHD), that appeared to be mainly catalyzed by P450 3A4 (*8*−*10*).

To know whether diclofenac, or one of its close analogues, could be used as tools for comparing the substrate selectivity and reactivity of human liver cytochromes P450, we have synthesized two diclofenac derivatives without carboxylate function, and compared their oxidation with various recombinant human P450s expressed in yeast to that of diclofenac itself. We have also studied their oxidation with chemical model systems of P450. Our results show that, in a general manner, diclofenac derivatives are mainly oxidized at position 5, their most reactive nucleophilic position. However, oxidation of diclofenac itself with P450 2C9, and to a latter extent, with P450 2C19 and 2C18, exibits a different regioselectivity with the exclusive (or major) formation of 4′-OHD. This particular regioselectivity should be due to a specific positioning of diclofenac in the P450 2C9 active site, because of a selective interaction of its COO^- function with a protein amino acid residue. These data suggest that diclofenac derivatives could be interesting probes for the knowledge of the active sites of P450 2Cs.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest quality commercially available. Diclofenac was purchased from Sigma. 3′-Hydroxy-, 4′-hydroxy-, and 5-hydroxy-diclofenac were a gift from Ciba-Geigy (Basel, Switzerland). Fe[TDCPP = *meso*-tetra(2,6-dichlorophenyl)porphyrin]Cl was prepared as described previously (*11*).

Physical Measurements. UV−vis spectra were performed on a Kontron Uvikon 820 spectrophotometer equipped with a diffusion accessory. ^{1}H NMR spectra were recorded at 27 °C on a Bruker ARX-250 instrument; chemical shifts are reported downfield from $(CH_3)_4Si$ in ppm, and *J* are in hertz. Labeling of the carbons of diclofenac derivatives corresponding to the following H assignments is given in Figure 1. Abbreviations used for peak description are s, d, t, m, bs, and dd for singlet, doublet, triplet, massif, broad singlet, and doublet of doublets, respectively. Elemental analyses were carried out at Centre Regional de Microanalyse, Paris.

*Synthesis of Diclofenac Derivatives. 2-[2-{(2,6-Diclorophenyl)amino}]phenyl ethanol, **2***. A solution of 630 mg of sodium diclofenac in 8 mL anhydrous, deaerated THF was added dropwise and under argon to a suspension of 200 mg of $LiAlH_4$ in 4 mL THF at 0 °C. After 2 h reaction under reflux of THF and cooling to 0 °C, 0.2 mL H_2O, 0.1 mL 10 M NaOH, and 0.2 mL H_2O were added successively. Diethyl ether was then added to the mixture and the precipitate was filtered on $MgSO_4$ and washed with diethyl ether. After solvent evaporation, purification by column chromatography (SiO_2, CH_2Cl_2), and crystallization from a 1:1 mixture of pentane and diethyl ether, 175 mg of **2** were obtained (31% yield). $F = 108$ °C. ^{1}H NMR ($CDCl_3$): 1.8 (t, 1H, OH), 3.01 (t, 2H, CH_2Ar), 4.01 (q, 2H, CH_2OH), 6.46 (d, 1H, H_3), 6.73 (bs, 1H, NH), 6.92 (dt, 1H, $J = 7.6$ and 1.6, H_5), 7.06 (dt, 1H, $J = 7.6$ and 1.6, H_4), 7.18 (dd, 1H, $J = 7.6$ and 1.6, H_6), 7.32 (d, 2H, $J = 8$, $H_{3'}$), and 6.95 (t, 1H, $J = 8$, $H_{4'}$). Anal. Calcd for $C_{14}H_{13}Cl_2NO$: C, 59.59; H, 4.64; N, 4.96. Found: C, 59, 59; H, 4.60; N, 5.00.

*3-[2-(2,6-Dichlorophenyl)amino]benzyloxy]propan-1-ol, **1**. 3-(Tetrahydropyranyloxy)-propan-1-ol:* a mixture of 1,3-propanediol (1 g), 3,4-dihydro-2*H*-pyrane (1 g), and *p*-toluene sulfonic acid (10 mg) in 1.25 mL anhydrous THF was stirred overnight. After neutralization with 20 μL pyridine, the solvent was evaporated and the sample was purified by column chromatography (SiO_2, cylohexane/ethyl acetate 1:1), yielding 900 mg of a colorless oil. *1-(2-iodobenzyloxy)-3-tetrahydropyranyloxy propane:* a solution of 480 mg of the previous alcohol in 0.5 mL THF containing one crystal of imidazole was added to 80 mg of NaH in 1 mL THF at 0 °C and under argon. After the reaction mixture was stirred for 1 h at room temperature, 28 mg of tetrabutylammonium iodide and 800 mg of 2-iodobenzyl chloride were added successively. After one night at 20 °C and 1 h at 50 °C, the reaction mixture was cooled to 20 °C and treated with 3 mL methanol. The residue obtained after solvent evaporation was dissolved in CH_2Cl_2, the solution washed with H_2O, dried over $MgSO_4$, and purified by column chromatography (SiO_2, CH_2Cl_2/diethyl ether 95:5). This led to 520 mg of a pale yellow oil (46% yield). Anal. Calcd for $C_{15}H_{21}IO_3$: C, 47.89; H, 5.63. Found: C, 47.80; H, 5.65. *1-{2-[(2,6-Dichlorophenyl)amino]benzyloxy-3-tetrahydropyranyloxy propane:* A mixture of the previous iododerivative (500 mg), 2,6-dichloroaniline (800 mg), anhydrous sodium carbonate (550 mg), and CuI (470 mg) in 10 mL toluene was heated to reflux in a Dean−Stark apparatus, containing molecular sieves (4 Å) for 4 days. After the reaction was cooled, filtrated, and the solvent evaporated, the residue was purified by column chromatography (SiO_2, CH_2Cl_2). The expected product was obtained in a 19% yield; its ^{1}H NMR spectrum was in complete agreement with the expected structure. *3-{2-[(2,6-Dichlorophenyl)amino]benzyloxy}propane-1-ol, **1**:* the previous compound (100 mg) was stirred overnight in 10 mL methanol, containing 11 mg of *p*-toluene sulfonic acid, at room temperature. Evaporation of the solvent and purification of the residue by column chromatography (SiO_2, CH_2Cl_2/ethyl acetate 95:5) led to 47 mg of **1**. ^{1}H NMR ($CDCl_3$): 1.9 (m, 3H), 3.69 (t, 2H, CH_2O), 3.78 (m, 2H, CH_2OH), 4.66 (s, 2H, CH_2Ar), 6.42 (d, 1H, H_3), 6.85 (m, 1H, H_5), 6.95 (bs, 1H, NH), 7.02 (t, 1H, $H_{4'}$), 7.10−7.20 (m, 2H, H_4 and H_6), 7.35 (d, 2H, $H_{3'}$). Anal. Calcd for $C_{16}H_{16}Cl_2O_2N$: C, 59.09; H, 4.96: N, 4.31. Found: C, 58. 96; H, 5.10; N, 4.34.

Yeast Transformation, Cell Culture, and Preparation of the Yeast Microsomal Fractions. The expression system used for human cytochromes P450 was based on a yeast strain W(R) fur 1 previously described (*12*), in which yeast

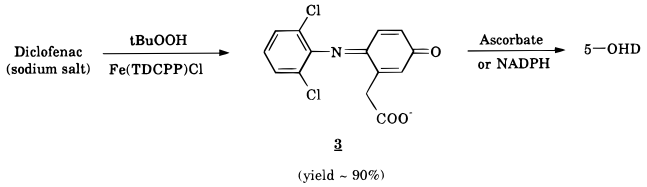
cytochrome P450 reductase was overexpressed. Transformation by a pYeDP60 vector containing one of the human liver CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 cDNAs (*13*−*16*) was then performed according to a general method of construction of yeast strain W(R) fur 1 expressing various human liver P450s (*17*, *18*). Yeast culture and microsomes preparation were performed by using previously described techniques (*19*). Microsomes were homogenized in 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA and 20% glycerol (v/v); the mixture was 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, 40, 10, and 200 pmol P450 per mg protein for CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4, respectively. Activities of CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 in yeast microsomes were, respectively, 10 (ethoxyresorufin O-deethylation), 2 (ethoxyresorufin O-deethylation), 0.2, 1.9, and 1.8 (5-hydroxylation of tienilic acid), 2 (4′-hydroxylation of S-mephenytoin), 5 (demethylation of dextromethorphan), 4 (6-hydroxylation of chlorzoxazone), and 1.8 (6β-hydroxylation of testosterone) nmol (nmol P450)$^{-1}$ min^{-1}.

Microsomal P450 content was determined according to Omura and Sato (*20*). The protein content in microsomal suspensions was determined by the Lowry procedure (*21*) using bovine serum albumin as standard.

Enzyme Activity Assay. Specific Activities of the Different P450s. Specific assays for CYP 1A1 and 1A2 (ethoxyresorufin O-deethylation (*22*)), 2C8, 2C9, and 2C18 (tienilic acid 5-hydroxylation (*23*)), 2C19 (*S*-mephenytoin 4′-hydroxylation (*24*)), 2D6 (dextromethorphan demethylation (*25*)), 2E1 (chlorzoxazone 6-hydroxylation (*26*)), and 3A4 (testosterone 6β-hydroxylation (*27*)) were carried out by using previously reported procedures.

Oxidation of Diclofenac Derivatives with Microsomes from Yeasts Expressing Human P450s. Diclofenac (sodium salt) (or its derivative) was incubated at 28 °C in the presence of yeast microsomes, containing 0.4 μM P450 (0.2 μM in the case of P450 2C9) in 200 μL of 20 mM sodium phosphate buffer, pH 7.4, containing 8% glycerol and a NADPH generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase per mL). To ensure adequate temperature equilibration, the microsomal incubation mixture and the solution containing the NADPH-generating system were first separately incubated for 3 min at 28 °C. Reaction was initiated with addition of the NADPH-generating system to the microsomal incubation mixture. It was stopped by cooling on ice and addition of 50 μL of an acetonitrile/acetic acid mixture (9:1). Proteins were precipitated by centrifugation for 5 min at 10 000 rpm and the supernatant stored at −40 °C until analysis.

Formation of the metabolites was followed by reverse phase HPLC (Spectra system AS 3000 autosampler). Supernatant aliquots were injected onto a Kromasil C18 column (ColoChrom, Gagny, France) (250 × 4.6 mm, 5 μm). The mobile phase (20 mM phosphate buffer (pH 7.4)/acetonitrile, gradient 20% up to 50% in 40 min) was delivered at a rate of 1 mL per min with a Spectra system P4000 pump. Monitoring of the column effluent was performed with a scanning Spectra Focus UV detector between 250 and 320 nm.



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(yield ~ 90%)

FIGURE 2: Oxidation of diclofenac with a biomimetic chemical model for cytochrome P450.

UV−*Visible Characteristics of Diclofenac Metabolites*. λ (ϵ in M^{-1} cm^{-1}) in 20 mM phosphate buffer, pH 7.4, were 280 (8480), 270 (8260), 281 (7470), and 284 (8950) for diclofenac and its 4′-, 3′-, and 5-hydroxy metabolites, respectively.

Oxidation of Diclofenac with Biomimetic Chemical Systems. A solution (0.7 mL) of *tert*-butylhydroperoxide (70% in H_2O) was progressively added to a mixture of diclofenac (sodium salt) (100 mg) and Fe(TDCPP)Cl (3.3 mg) in 1.6 mL of $CH_3CN/H_2O/CH_2Cl_2$ (1:0.5:0.1 v/v), at room temperature. After total consumption of diclofenac, as shown by HPLC analysis, the reaction mixture was poured in 40 mL H_2O. After extraction with 10 mL ethyl acetate, lyophilization of the aqueous phase led to 90 mg of quinoneimine **3** (sodium salt). Its UV−vis spectrum (in H_2O): λm (ϵ in M^{-1} cm^{-1}) = 471 (740) and 270 (13 500) nm. ^{1}H NMR spectrum (D_2O): 6.9 (d, $J_{34} = 10$, H_3), 6.6 (dd, $J_{43} = 10$ and $J_{46} = 2.2$, H_4), 6.7 (d, $J_{64} = 2.2$, H_6), 7.25 (t, $J = 8$, $H_{4'}$), 7.55 (d, $J = 8$, $H_{3'}$), and 3.7 (s, CH_2), and easy transformation into 5-OH-diclofenac upon treatment with NADPH, sodium ascorbate or sodium borohydride, are in complete agreement with the structure **3** indicated in Figure 2.

*Oxidation of the Diclofenac Derivative **2** with Biomimetic Chemical Systems*. A solution (51 μL) of *tert*-butylhydroperoxide (70% in H_2O) was progressively added over 1.5 h at 20 °C to a mixture of **2** (10 mg) and Fe(TDCPP)Cl (1 mg) in 0.3 mL CH_2Cl_2. After the mixture was stirred overnight and purified by HPLC, quinone **4** was obtained in a 75% yield. ^{1}H NMR ($CDCl_3$): $\delta = 6.6$ (d, $J = 10$, H_3), 6.45 (dd, $J = 10$ and 2, H_4), 6.64 (m, H_6), 7 (t, $J = 8$, $H_{4'}$), 7.37 (d, $J = 8$, $H_{3'}$), 4 (t, $J = 6$, CH_2), and 3 (t, $J = 6$, CH_2O), is in complete agreement with the proposed structure. Reduction of **4** by sodium borohydride led to alcohol **5** in an almost quantitative yield. The HPLC retention time and ^{1}H NMR ($CDCl_3$) spectrum of that compound: $\delta = 6.4$ (d, $J = 8.6$, H_3), 6.55 (dd, $J = 8.6$ and 2.9, H_4), 6.7 (d, $J = 2.9$, H_6), 6.9 (t, $J = 8$, $H_{4'}$), 7.3 (d, $J = 8$, $H_{3'}$), 4 (t, $J = 5.8$, CH_2O), and 2.98 (t, $J = 5.8$, CH_2), are identical to those observed for the main metabolite derived from the oxidation of **2** with recombinant human P450 2Cs.

RESULTS

Oxidation of Diclofenac by Recombinant Human Liver P450s. Oxidations of diclofenac were performed by using microsomes of yeasts expressing a given human P450 in the presence of NADPH. Yeasts were obtained by transformation of yeast strain W(R)fur 1, in which P450 reductase was overexpressed (*12*), with a pYeDP60 vector containing the cDNA of a given human P450 (*13*−*18*). For each recombinant P450, we have checked that the formation of the various metabolites was linear as a function of time for at least 10 min, and as a function of P450 concentration in the

Table 1: Kinetic Data for the Hydroxylation of Diclofenac with Microsomes from Yeast Expressing Various Human Liver P450s[a]

product	P450	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1}\ \mu M^{-1}$)
4′-OHD	2C9	24 ± 2	15 ± 8	1.6
	2C8	1.2 ± 0.2	630 ± 30	0.0019
	2C18	1.1 ± 0.2	170 ± 30	0.006
	2C19	1.4 ± 0.5	440 ± 50	0.003
5-OHD	2C8	7 ± 1	280 ± 30	0.025
	2C18	3 ± 0.4	125 ± 20	0.024
	2C19	1.1 ± 0.5	470 ± 50	0.002

[a] Conditions indicated in Materials and Methods. Results are mean values ± SD from 3 to 6 experiments.

0.1−1 μM range. We also checked that these metabolites were not formed in incubations not containing NADPH as well as in incubations using microsomes of yeast strain W(R)fur 1 transformed with the vector not containing any human liver cDNA.

HPLC studies of incubates of diclofenac with microsomes from yeast expressing P450 2C9 in the presence of NADPH showed the time-dependent formation of only one metabolite that exhibits a retention time and UV spectrum identical to those of an authentic sample of 4′-hydroxy diclofenac (4′-OHD), in agreement with previous data on the same reaction catalyzed by human liver microsomes (*6*, *7*). Treatment of the kinetic data by using classical Lineweaver−Burk plots led to k_{cat} and K_M values of 24 ± 2 min^{-1} and 15 ± 8 μM (Table 1) for this CYP 2C9-dependent 4′-hydroxylation of diclofenac.

Similar experiments performed with microsomes from yeast expressing P450 2C19 led to a more complex HPLC pattern that showed the simultaneous formation of two metabolites. The HPLC retention times and UV spectra of these metabolites were found identical to those of authentic samples of 5-hydroxy- and 4′-hydroxy-diclofenac (5-OHD and 4′-OHD). P450 2C19 led to similar amounts of 4′-OHD and 5-OHD (in a 55:45 molar ratio). It is not only less regioselective than P450 2C9, but also much less efficient for the hydroxylation of diclofenac. This is shown by the k_{cat}/K_M values determined for the P450 2C19-catalyzed 4′- and 5-hydroxylation of diclofenac that are two to 3 orders of magnitude lower than the one found for P450 2C9-catalyzed 4′-hydroxylation of diclofenac (3×10^{-3} and 2×10^{-3} instead of 1.6 $min^{-1}\ \mu M^{-1}$) (Table 1). This large difference is due to the apparent K_M values for P450 2C19-catalyzed 4′- and 5-hydroxylation of diclofenac (440 ± 50 and 470 ± 50 μM, respectively), which are much higher than that found for P450 2C9-dependent 4′-hydroxylation of diclofenac (15 ± 8 μM), and to the k_{cat} values for those P450 2C19 reactions (1.4 and 1.1 ± 0.5 min^{-1}) that are markedly lower than the one measured for P450 2C9 (24 ± 2 min^{-1}).

Recombinant P450 2C18 also led to a mixture of 4′-OHD and 5-OHD, 5-OHD now being the major metabolite (approximate value of 25:75 for the 4′-OHD/5-OHD molar ratio). The k_{cat}/K_M values measured for these hydroxylations (6×10^{-3} and 24×10^{-3} $min^{-1}\ \mu M^{-1}$) were slightly higher than those found for the corresponding P450 2C19-catalyzed reactions but remained much lower than that found for P450 2C9 (Table 1).

Recombinant P450 2C8 mainly led to the formation of 5-OHD and only to minor amounts of 4′-OHD. Its catalytic efficiency was also found to be much lower than that of P450 2C9 (k_{cat}/K_M values for P450 2C8-catalyzed 5- and 4′-hydroxylation of diclofenac: 25×10^{-3} and 2×10^{-3} $min^{-1}\ \mu M^{-1}$) (Table 1).

Microsomes from yeast expressing P450 3A4 only led to the formation of 5-OHD but in low amounts that were difficult to measure in a quantitative manner. Supplementary experiments using commercially available microsomes from insect cells infected with baculovirus and coexpressing P450 3A4 and human P450 reductase in large excess (Gentest Corporation, USA) confirmed the almost exclusive formation of 5-OHD.

Microsomes from yeast expressing P450 1A1, 1A2, 2D6, and 2E1 failed to give any detectable hydroxylated metabolite even when using 500 μM diclofenac.

Oxidation of Diclofenac by Chemical Systems Mimicking Cytochromes P450. To interpret the regioselectivity of the hydroxylation of diclofenac by the different human P450s, it was interesting to know the regioselectivity of the oxidation of diclofenac by chemical model systems able to generate iron active species that mimic the reactivity of high-valent P450-iron-oxo intermediates. Chemical systems using Fe(III) or Mn(III) porphyrins as catalysts and oxygen atom donors, such as PhIO, H_2O_2, tBuOOH, or O_2 in the presence of a reducing agent, are well-known to reproduce the main reactions of P450s. Thus, these systems catalyze the epoxidation of alkenes, hydroxylation of C−H bonds, hydroxylation of aromatic rings as well as S-oxidation of thioethers and N-oxidation of amines (*28*−*30*).

Oxidation of diclofenac by the P450 model system using Fe(III) [TDCPP = *meso*-tetra(2,6-dichlorophenyl)porphyrin]-Cl as catalyst and tBuOOH as oxygen atom donor led to the formation of only one product with an almost quantitative yield. Isolation of this product and its characterization by UV and ^{1}H NMR spectroscopy showed that it was the quinone-imine **3** derived from two-electron oxidation of 5-OHD. Accordingly, treatment of this product with a reducing agent, ascorbate or NADPH, led to its quantitative transformation into 5-OHD (Figure 2). The easy oxidation of 5-OHD into the corresponding quinone-imine and reduction of the latter into 5-ODH with ascorbate were recently reported (*8*).

These results show that chemical oxidation of diclofenac with species similar to those involved in P450-dependent monooxygenases mainly leads to products deriving from oxidation at position 5. This chemoselectivity is easily understandable if one considers that the diclofenac aryl ring bearing the electron-donating CH_2COO^- substituent is more electron-rich and more reactive toward electrophilic iron-oxo species than the dichlorophenyl ring. Moreover, the 5-position of diclofenac should be more reactive as it is para to the electron-donating NH substituent. Therefore, it is not surprising that oxidation of diclofenac with the iron-porphyrin electrophilic species mainly occurs at position 5, provided that diclofenac has a free access to the iron active species.

The completely different regioselectivity observed during P450 2C9-catalyzed oxidation of diclofenac, which leads to the hydroxylation of the less electron-rich aromatic ring (4′-hydroxylation), should thus be due to a particular positioning of the molecule relative to P450 2C9-iron-oxo active species. It is tempting to propose that this positioning is imposed by the binding of the COO^- function of diclofenac to an amino

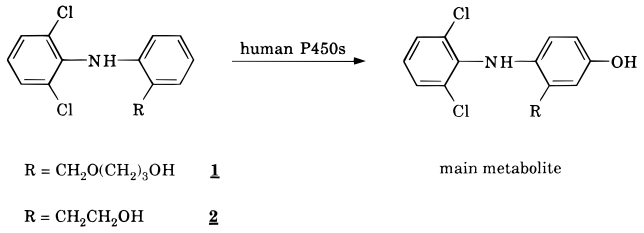

FIGURE 3: Main metabolites derived from the oxidation of compounds **1** and **2** with recombinant human liver P450s.

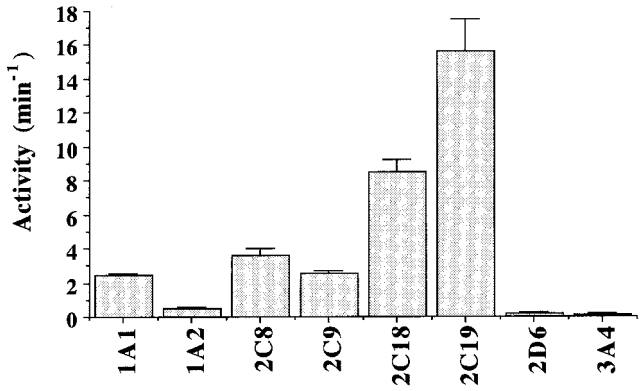

FIGURE 4: 5-Hydroxylase activities of various recombinant human liver P450s toward compound **2**. Conditions indicated in Materials and Methods; activities are initial rates of 5-hydroxylation in nmol 5-hydroxy metabolite. (nmol P450)$^{-1}$ min^{-1} observed for incubations with 30 μM **2**.

acid residue of the protein. Models of P450 2C9 active site in which anionic P450 2C9 substrates are bound to a cationic amino acid residue of the protein have been published recently (*31*−*36*).

To test this hypothesis, two diclofenac derivatives not involving this COO^- function, **1** and **2** (Figure 3), have been synthesized and oxidized by yeast microsomes expressing human P450s. Oxidation of compounds **1** and **2** with microsomes from yeasts expressing P450 2C9, 2C8, or 2C18 led, in all cases, to a major metabolite whose UV and ^{1}H NMR spectra clearly indicated that it derived from the 5-hydroxylation of **1** and **2**. Their UV (λm $\approx$ 280 nm) and ^{1}H NMR (aromatic protons signals) characteristics were almost identical to those of 5-OHD. Since the regioselectivity of CYP 2C-dependent hydroxylations of **1** and **2** appeared to be very similar, further detailed experiments were only done on compound **2**.

*Oxidation of Diclofenac Derivative **2** by Recombinant Human Liver P450s*. After 30 min incubations using 100 μM **2** and 0.2 μM P450, all the P450s tested (1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, and 3A4) led almost exclusively to the formation of the 5-hydroxy metabolite. In the case of P450 2C18 and 2C19, a minor metabolite (5−10% relative to the major one) was detected by HPLC. It showed an UV spectrum very similar to that of 4′-OHD; however, it was formed in too low amounts for further characterization. Under the aforementioned conditions, P450 2C18 and 2C19 were at least 10 times more active than the other P450s used (rates between 150 and 220 nmol 5-hydroxy metabolite nmol P450^{-1}.30 min^{-1} instead of 5 to 25 for the other P450s).

Figure 4 illustrates the higher efficiency of P450s of the 2C subfamily to catalyze the 5-hydroxylation of **2**, in incubations using 30 μM **2**. If one excepts P450 1A1 that exhibited an initial rate of 2 min^{-1}, then only the P450 2Cs showed significant initial rates (between 2 and 16 min^{-1}) for this reaction. Table 2 compares the kinetic parameters of 5-hydroxylation of **2** by CYP 2C8, 2C9, 2C18, and 2C19. All P450 2Cs, except P450 2C9, are characterized by relatively large k_{cat} around 25 min^{-1}. P450 2C18 appears as the most efficient enzyme with a k_{cat}/K_M value of 1.6 min^{-1} μM^{-1}; however, the k_{cat}/K_M values for the four P450 2Cs only varied between 0.2 and 1.6 min^{-1} μM^{-1}.

Table 2: Kinetic Data for the 5-Hydroxylation of Compound **2** by Recombinant Human P450 2Cs[a]

P450	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})
2C8	26 ± 8	130 ± 10	0.2
2C9	2.5 ± 1	5 ± 2	0.5
2C18	21 ± 3	13 ± 4	1.6
2C19	29 ± 5	54 ± 11	0.5

[a] Conditions indicated in Materials and Methods. Results are mean values ± SD from 3 experiments.

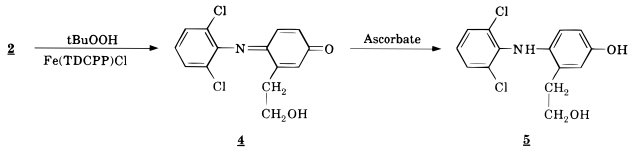

FIGURE 5: Oxidation of compound **2** with a biomimetic chemical model for cytochrome P450.

*Oxidation of **2** with Chemical Model Systems*. Oxidation of **2** with tBuOOH in the presence of catalytic amounts of Fe(TDCPP)Cl led to the formation of almost only one product. The UV and ^{1}H NMR spectra of this product were very similar to those of the quinone-imine **3** derived from chemical oxidation of diclofenac (see above) (at least for the NMR signals of the aromatic protons). Moreover, reduction of this product with sodium ascorbate led to a new compound showing a UV spectrum and HPLC retention time identical to 5-hydroxy-**2** (Figure 5). Thus, oxidations of **2** and diclofenac with chemical models of P450 exhibit an identical regioselectivity greatly in favor of position 5.

DISCUSSION

Oxidation of Diclofenac Derivatives with P450s and Chemical Models Generally Occurs at Position 5. Oxidation of diclofenac and its derivative **2** with chemical systems based on Fe(III) porphyrins, which reproduce the oxidation reactions catalyzed by P450s, mainly leads to products deriving from an oxidation at position 5 of these molecules (Figure 2 and 5).

Position 5, para to the NH group on the more electron-rich aromatic ring of diclofenac derivatives, thus appears as the privileged site of oxidation after reaction with electrophilic, oxidant species. This is in agreement with previous data reported on the oxidation of diclofenac with ClO^- (*37*). In a general manner, human P450s exhibit a similar tendency to hydroxylate diclofenac derivatives mainly at position 5. This was found for all the P450s tested in the case of compounds **1** and **2**. Diclofenac itself was also mainly hydroxylated at position 5 by all P450s tested except for P450 2C9 and 2C19, even though the corresponding catalytic efficiencies remained low (k_{cat}/K_M values lower than 0.03 min^{-1} μM^{-1}).

The detailed mechanisms of introduction of an hydroxy (or oxo) group at position 5 of diclofenac remain to be determined, and are out of the scope of this publication. Such mechanisms could involve either an hydroxylation at position 5 of diclofenac or a N-oxidation of diclofenac, with appearance of a cationic or radical intermediate at carbon 5 within a quinone-imine type species, and transfer of an OH group to this intermediate.

The Peculiar Behavior of Human P450 2Cs in the Oxidation of Diclofenac Derivatives. P450 2Cs are the most efficient human liver isozymes for the oxidation of diclofenac derivatives. This is true for the 5-hydroxylation of compound **2** (Figure 4) and for the oxidation of diclofenac. Moreover, P450 2Cs exhibit a peculiar regioselectivity in their hydroxylation of diclofenac itself, that is clearly related to the presence of a COO^- function. This is particularly striking in the case of P450 2C9, which selectively hydroxylates **2** at position 5, whereas it hydroxylates diclofenac exclusively at position 4′. This is also true in the case of P450 2C18 and 2C19 which hydroxylate **2** mainly at position 5 whereas they oxidize diclofenac at position 4′ and 5.

Diclofenac hydroxylation patterns may thus be used as fingerprints for human P450 2Cs. Exclusive formation of 4′-OHD with a very high catalytic efficiency ($k_{cat}/K_M \sim 2$ min^{-1} μM^{-1}) is distinctive of P450 2C9. By contrast, P450 2C19, 2C18, and 2C8 hydroxylate diclofenac at both positions 5 and 4′ with much lower efficiencies (k_{cat}/K_M between 2×10^{-3} and 3×10^{-2} min^{-1} μM^{-1}). Moreover, the 4′-OHD/5-OHD molar ratio is different for each P450 2C; it varies from 0.17, for P450 2C8, to 0.37, for P450 2C18, and 1.3, for P450 2C19.

Consequences of the Above Results for the Topology of Human P450 2Cs Active Sites. As far as the topology of P450 2Cs active sites is concerned, the aforementioned results indicate that P450 2C9 imposes a strict positioning of diclofenac in the active site in order to completely orientate its hydroxylation toward position 4′ that is not chemically preferred. This positioning should mainly involve the COO^- function as compounds **1** and **2** lacking this function are hydroxylated at the chemically preferred position 5. This could be due to the strong binding of this anionic function to a cationic amino acid residue of the protein, as previously proposed (*31*−*33*). The other human P450s tested would not involve such a strong structural recognition determinant, as they leave a sufficient mobility of diclofenac in their active site to permit its chemically preferred oxidation at position 5. P450 3A4 selectively leads to 5-OHD, whereas P450 2C8, 2C18, and 2C19 lead to a mixture of 5-OHD and various amounts of 4′-OHD (up to 55% in the case of P450 2C19). It is thus tempting to speculate that positioning of diclofenac by amino acid residues of the P450 2C8, 2C18, and 2C19 active sites is strong enough to favor hydroxylations at positions different from the chemically preferred position 5, even though this positioning is less strict than in P450 2C9 active site.

Moreover, a comparison of the kinetic values found for hydroxylation of diclofenac with the P450s of the 2C subfamily clearly shows that the catalytic efficiency of P450 2C9 is much higher than those of the three other 2C isozymes (Table 1). This appears to be mainly due to marked increases of the K_M values, which suggests a much better affinity of diclofenac for P450 2C9 than for the other P450 2Cs. It is likely that this better affinity of diclofenac for P450 2C9 is due to the strong binding of its COO^- group to a cationic residue of the protein. P450 2C19 does not seem to act as a good catalyst for compounds containing a COO^- function, as shown by its very low efficiency to oxidize diclofenac (this work and ref *38*), and by previous literature data showing that it is inactive for tienilic acid (*23*) and ibuprofen (*38*) oxidation. In a more general manner, the lack of inhibitory effects of sulfaphenazole toward P450 2C19-catalyzed reactions (*7*, *39*) indicates that this P450 has a low affinity for anionic molecules. P450 2C18 and 2C8 have also been shown to exhibit lower affinities than P450 2C9 for tienilic acid (*23*) and lower abilities to be inhibited by sulfaphenazole (*7*).

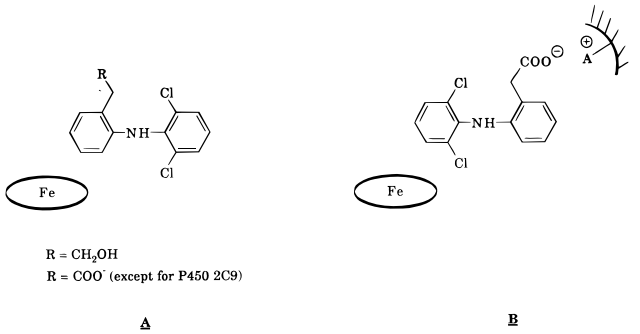

FIGURE 6: Schematic view of the two possible positionings of diclofenac derivatives in P450 active sites: A, situation found with diclofenac and all active P450s except P450 2C9, and with **2** and all P450s tested; B, situation found for diclofenac and P450 2C9.

In a schematic manner, our results suggest two extreme situations depicted in Figure 6 for the positioning of diclofenac derivatives in P450 active sites: (i) the first one would correspond to the chemically preferred oxidation with the arylacetic ring close to the active Fe═O species, and is found in most cases (substrates **1** and **2** and all active P450s; diclofenac and many P450s) (Figure 6A) and (ii) the second situation would correspond to the existence of a strong interaction between the COO^- group of diclofenac and a P450 residue. It is the only one found with P450 2C9, and it partly occurs in the case of P450 2C19, 2C18, and 2C8 (Figure 6B).

Anyway, the above results show that diclofenac and its derivatives are interesting tools to study the differences between the active site topologies of human P450 2Cs.

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