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ROLE OF CYTOCHROME P450 2C9 AND AN ALLELIC VARIANT IN THE 4′-HYDROXYLATION OF (*R*)- AND (*S*)-FLURBIPROFEN

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Abstract—Flurbiprofen is a chiral non-steroidal anti-inflammatory drug used in the treatment of pain or inflammation. The primary routes of biotransformation for (*R*)- and (*S*)-flurbiprofen are oxidation (presumably cytochrome P450) and conjugation. To date, the specific cytochrome P450 (P450) involved in the oxidative metabolism of this compound (specifically 4′-hydroxylation) has not been elucidated. Experiments were conducted to characterize the kinetic parameters (K_m and V_{max}) for the 4′-hydroxylation of (*R*)- and (*S*)-flurbiprofen in human liver microsomes, to determine if enantiomeric interactions occur when both enantiomers are present, and to identify the specific P450 form(s) involved in this reaction. In human liver microsomes, the K_m and V_{max} (mean ± SD) for (*R*)-4′-hydroxy-flurbiprofen formation were 3.1 ± 0.8 μM and 305 ± 168 pmol·min^{-1}·mg protein)$^{-1}$, respectively. In comparison, the K_m and V_{max} (mean ± SD) for (*S*)-4′-hydroxy-flurbiprofen formation were 1.9 ± 0.4 μM and 343 ± 196 pmol·min^{-1}·mg protein^{-1}, respectively. Enantiomeric interaction studies revealed a decrease in K_m and V_{max} for both enantiomers and an apparent loss of stereoselectivity. *Racemic*-warfarin, tolbutamide, α-naphthoflavone and erythromycin were studied as potential inhibitors of this process. The estimated K_i values for the inhibition of (*R*)- and (*S*)-4′-hydroxy-flurbiprofen formation by *racemic*-warfarin were 2.2 and 4.7 μM. This reaction was also inhibited by tolbutamide. In contrast, erythromycin and α-naphthoflavone had no appreciable effect on 4′-hydroxy-flurbiprofen formation. cDNA expression of individual forms was used to determine which P450 was involved in 4′-hydroxy-flurbiprofen formation. P450 2C9 and an allelic variant (R144C) readily catalyzed the formation of 4′-hydroxy-flurbiprofen. P450 1A2 was also active albeit with a turnover rate 1/140th that of P450 2C9R144C (P450s 2C8, 2E1 and 3A4 were not active toward either enantiomer). The results of these studies indicate that the enantiomers of flurbiprofen may exhibit stereoselectivity with respect to enzyme affinity but have roughly equal maximum formation velocities. Additionally, these two enantiomers may compete for the enzyme resulting in lower maximum velocities for both enantiomers. Finally, of those P450 forms examined, only P450 2C9 and an allelic variant catalyzed the 4′-hydroxylation of both (*R*)- and (*S*)-flurbiprofen.

Key words: flurbiprofen; cytochrome P450; human liver microsomes; vaccinia virus; cDNA expression; enantiomers

Flurbiprofen (*racemic*-2-[2-fluoro-4-biphenyl]propionic acid) is an NSAID‖ used in the treatment of pain or inflammation [1]. Flurbiprofen belongs to the chemical class of NSAIDs known as the "profens" and is administered as a racemic mixture. Like its structural analogs ibuprofen and fenoprofen, only the (*S*)-enantiomer of flurbiprofen is active in inhibiting cyclooxygenase [2], and thus its therapeutic effect is dependent on the elimination of this enantiomer. However, unlike the structural analogs, flurbiprofen does not undergo metabolic inversion of configuration [3]. The flurbiprofen enantiomers are oxidized to form 4′-hydroxy-flurbiprofen and 3′,4′-dihydroxy-flurbiprofen or are further methylated to form 3′-hydroxy-4′-methoxy-flurbiprofen [4, 5]. These metabolites, as well as the parent compounds, can then be conjugated by either glucuronidation or sulfation [4, 5]. With respect to oxidation, the formation of 4′-hydroxy-flurbiprofen (Fig. 1) accounts for 86% of the oxidative metabolites [6] and probably occurs via the P450 system.

To date, only *in vivo* studies have been conducted on the oxidative biotransformation of the flurbiprofen enantiomers [4, 5], and no specific P450 form has been attributed to performing this reaction. *In vivo* studies demonstrated a slight (0.8 *R*/*S* ratio) stereoselectivity in flurbiprofen metabolism, but these studies were based on urinary elimination data and thus may be influenced by other non-metabolic factors [4, 5]. Recently, Leemann *et al.* [7] reported that P450^{TB} (presumably equivalent to P450 2C8 and/or P450 2C9) is responsible for the hydroxylation of diclofenac, an NSAID like flurbiprofen, based

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‖ Abbreviations: NSAID, non-steroidal anti-inflammatory drug; and cDNA, complementary deoxyribonucleic acid.

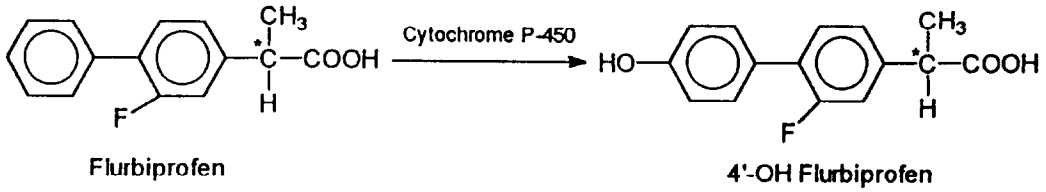

Fig. 1. Structures of flurbiprofen and its 4′-hydroxy-metabolite. An asterisk indicates the chiral center.

on chemical inhibition studies in human liver microsomes. Furthermore, diclofenac, indomethacin, piroxicam and pirprofen were reported to inhibit the hydroxylation of tolbutamide [8]. Therefore, studies with other NSAIDs would suggest that either P450 2C8 or P450 2C9 may be involved in the metabolism of flurbiprofen and that at least some of these oxidative processes may be stereoselective. However, it is still unknown whether the oxidation of both enantiomers is catalyzed by a single enzyme or whether the enantiomers could be oxidized by separate P450 forms.

The objectives of our study were to characterize the 4′-hydroxylation of (*R*)- and (*S*)-flurbiprofen in human liver microsomes, to determine whether this process was stereoselective and whether interactions between the two enantiomers occur, and to characterize the specific P450 forms responsible for the 4′-hydroxylation of the enantiomers of flurbiprofen. The results of these studies are reported herein.

MATERIALS AND METHODS

Chemicals and reagents. Ethyl acetate, hexane, acetonitrile and potassium phosphate were obtained from the Fisher Co. (Pittsburgh, PA). Carbonyldiimidazole and (*S*)-α-methylbenzylamine were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Tolbutamide and *racemic*-warfarin were obtained from the Sigma Chemical Co. (St. Louis, MO). (*R*)- and (*S*)-Flurbiprofen, 4′-hydroxy-flurbiprofen and 2-fluoro-4-biphenyl acetic acid were gifts of the Upjohn Co. (Kalamazoo, MI). All other chemicals were obtained from commercial sources and were of the highest purity available.

Human liver tissue and cDNA-transfected cells. Human liver tissue (N = 7) for preparation of microsomes was obtained under protocols approved at the Medical College of Wisconsin or through the Liver Tissue Procurement and Distribution System (LTPADS). Based on chart review, none of the patients from which tissues were obtained was taking any medications known to inhibit or induce the P450s. Microsomal samples were prepared according to established methods [9]. Protein content was measured by the method of Lowry *et al.* [10] and P450 content by the method of Omura and Sato [11].

Human HepG2 cells were infected with a recombinant vaccinia virus containing cDNAs for P450s 1A2, 2C8, 2C9, 2C9R144C, 2E1 and 3A4. The details of construction of the viruses were published previously [12]. Control cells were infected with wild type vaccinia virus. The cells were harvested 1 day after infection and stored frozen at −80°. Prior to use, the cells were thawed to 4°, spun to remove the supernatant, and resuspended in 50 mM K_2HPO_4, pH 7.4. The cells were then sonicated with two 5-sec bursts prior to addition to the incubation mixture.

Liver microsomal incubations. Liver microsomes (0.1 mg/mL final protein concentration) were incubated in the presence of 1 mM β-NADP, 10 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase and 100 mM K_2HPO_4, pH 7.4, in a total volume of 200 μL. Reactions were carried out for 20 min at 37° over the (*R*)- or (*S*)-flurbiprofen concentration range of 1–150 μM. The reaction was found to be linear over the tested protein concentration range of 0.05 to 0.4 mg/mL and over durations of 5–20 min. Experiments involving cDNA-expressed P450s were carried out at 12.5 μM (*R*)- or (*S*)-flurbiprofen concentrations under the same conditions except that the buffer was 50 mM K_2HPO_4, pH 7.4, 0.4 U glucose-6-phosphate dehydrogenase was added, and the total volume was 400 μL. A substrate concentration of 12.5 μM was chosen for the cDNA-expressed P450 experiments, since this concentration had produced maximal velocity in the formation of 4′-hydroxy-flurbiprofen in all human liver samples studied.

HPLC assay of 4′-hydroxy-flurbiprofen and its enantiomers. The measurement of 4′-hydroxy-flurbiprofen after incubation of either (*R*)- or (*S*)-flurbiprofen was carried out as follows. The microsomal incubation reaction was terminated by the addition of 500 μL of acetonitrile. To the samples, 9 ng of 2-fluoro-4-biphenyl acetic acid (internal standard), 20 μL of H_3PO_4 and 300 μL of H_2O were added. The samples were centrifuged at 11,000 *g* for 4 min and the supernatant was transferred to a clean tube. To the supernatant, 2 mL of ethyl acetate was added; samples were vortexed for 1 min and then centrifuged at 2000 *g* for 5 min. The upper organic layer was then removed and evaporated to dryness. Samples were then reconstituted in 150 μL of mobile phase, and 10–50 μL was injected onto the HPLC system. The HPLC system consisted of a Waters® 501 HPLC pump, a Waters® 717 autosampler and a Waters® 470 fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 320 nm. The mobile phase consisted of acetonitrile:10 mM K_2HPO_4, pH 3.0 (39:61), pumped at 1 mL/min through a Brownlee Spheri-5® C_{18} 4.6 × 100 mm column.

Analysis of the enantiomers of 4′-hydroxy-

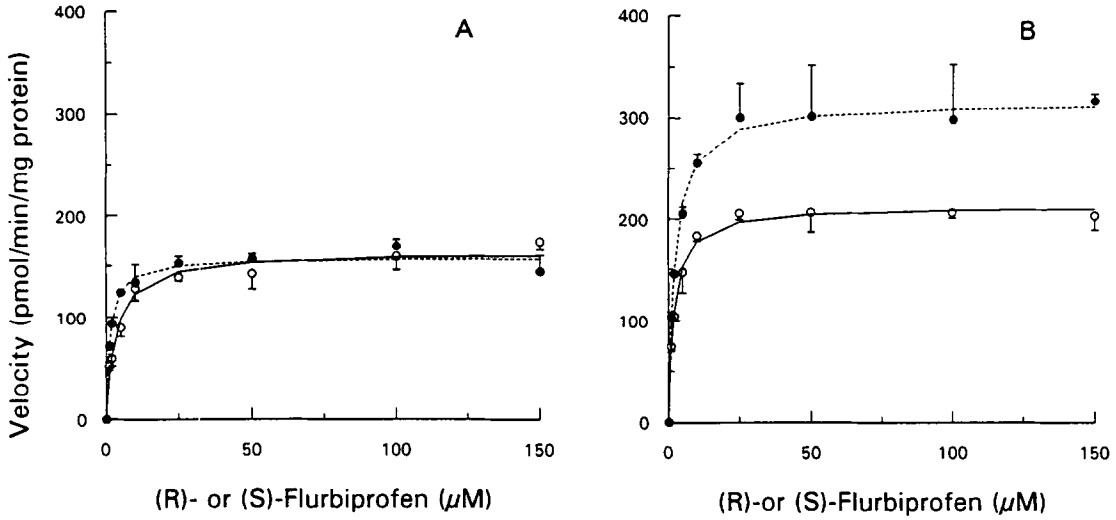

Fig. 2. Formation of either (*R*)- or (*S*)-4′-hydroxy-flurbiprofen from (*R*)- and (*S*)-flurbiprofen, respectively, in human liver microsomes. Panel A is the formation in a liver sample exhibiting no difference in V_{max}. Panel B is the formation in a liver sample exhibiting a difference in V_{max}. Key: (●) (*S*)-4′-hydroxy-flurbiprofen, and (○) (*R*)-4′-hydroxy-flurbiprofen. Dashed lines represent the nonlinear regression fits of (*S*)-4′-hydroxy-flurbiprofen data, and solid lines represent the nonlinear regression fits of (*R*)-4′-hydroxy-flurbiprofen data. Values are means ± SD of three determinations.

flurbiprofen following incubation of both (*R*)- and (*S*)-flurbiprofen simultaneously was carried out according to previously published methods [5] with the following modifications. 2-Fluoro-4-biphenyl acetic acid (45 ng) was added as internal standard, and the samples were extracted as described above. The samples were then derivatized with α-methylbenzylamine as described and washed only once with 0.5 M NH_4OH and 1 M HCl, respectively. The HPLC conditions were as described above except that the mobile phase composition was acetonitrile : water (49 : 51).

Data analysis. Data on 4′-hydroxy-flurbiprofen formation in human liver microsomes were analyzed by nonlinear regression (PCNONLIN, Statistical Consultants, Inc., Lexington, KY, U.S.A.) of the substrate concentration versus velocity plots according to the Michaelis–Menten equation. The effect of *racemic*-warfarin and tolbutamide on the formation of 4′-hydroxy-flurbiprofen was evaluated by estimating the apparent inhibition constant (K_i) for the inhibitors according to the methods of Cleland [13]. Statistical comparisons of K_m and V_{max} for the formation of 4′-hydroxy-flurbiprofen by (*R*)- and (*S*)-flurbiprofen were made using a paired *t*-test. Statistical significance was determined at $P \leq 0.05$.

RESULTS

Formation of 4′-hydroxy-flurbiprofen in a representative sample is depicted in Fig. 2A. However, in three of the seven liver samples, some stereoselectivity in V_{max} estimates was noted, and a representative tissue is depicted in Fig. 2B. When one compares the estimates of K_m and V_{max} for all seven samples (Table 1), it can be noted that the mean estimate of K_m when (*S*)-flurbiprofen (K_m = 1.9 μM) was used as a substrate was statistically different (albeit slight) from that observed when (*R*)-flurbiprofen (K_m = 3.1 μM) was a substrate for the reaction. However, no statistical difference could be noted in the mean estimates of V_{max} when either enantiomer was used as a substrate. Thus, there appears to be slight stereoselectivity in the affinity for the enantiomers of flurbiprofen but not with respect to the maximum velocity of the reaction.

Enantiomeric interaction studies were carried out to determine whether the enantiomers of flurbiprofen were being hydroxylated by the same P450, and, if so, whether these enantiomers would compete for this process as would be occurring *in vivo*. These studies were carried out in two of the previously studied samples for comparisons. Reactions were carried out over the same concentration ranges as when single enantiomers were used with the opposing enantiomer being present in equimolar concentrations (i.e. a racemic mixture resulting in double the *total* substrate present). Representative results of these studies are presented in Fig. 3. Estimates of K_m and V_{max} for the formation of each enantiomer of 4′-hydroxy-flurbiprofen when *racemic*-flurbiprofen was used as substrate are presented in Table 2 and can be compared with the single enantiomer studies presented in Table 1. It should be noted that the estimates of K_m and V_{max} were reduced in both cases, and it would appear that the stereoselectivity with respect to enzyme affinity (K_m) was no longer observed.

Initial attempts to determine the P450 forms involved in the reactions were conducted using chemical inhibition studies with putative specific

Table 1. Estimates of K_m and V_{max} for formation of (*S*)- and (*R*)-4′-hydroxy-flurbiprofen in human liver samples

	K_m [μM]		V_{max} [pmol·min^{-1}·(mg protein)$^{-1}$]	
Sample	(*S*)-4′OHF*	(*R*)-4′OHF†	(*S*)-4′OHF	(*R*)-4′OHF
1	1.8	3.1	729	581
2	1.3	3.3	159	164
3	2.0	4.3	149	104
4	2.4	2.0	317	213
5	2.4	3.1	386	389
6	2.0	3.7	267	255
7	1.4	2.5	396	429
Mean (SD)	1.9 (0.4)‡	3.1 (0.8)	343 (196)	305 (168)

* (*S*)-4′-Hydroxy-flurbiprofen.
† (*R*)-4′-Hydroxy-flurbiprofen.
‡ Statistically different from (*R*)-4′OHF at P = 0.01.

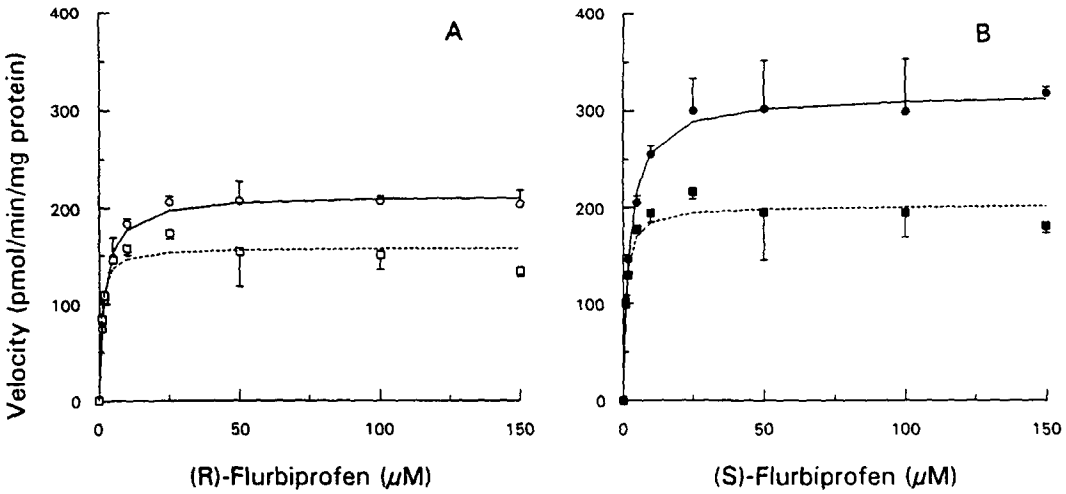

Fig. 3. Enantiomeric interaction studies involving (*R*)- and (*S*)-flurbiprofen in human liver microsomes. Panel A represents the formation of (*R*)-4′-hydroxy-flurbiprofen from (*R*)-flurbiprofen in the absence (○) and presence (□) of equimolar concentrations of (*S*)-flurbiprofen. Solid and dashed lines represent the nonlinear regression fits of the absence and presence of inhibitor data, respectively. Panel B represents the formation of (*S*)-4′-hydroxy-flurbiprofen in the absence (●) and presence (■) of equimolar (*R*)-flurbiprofen. Solid and dashed lines represent the nonlinear regression fits of the absence and presence of inhibitor data, respectively.

Table 2. Estimates of K_m and V_{max} for (*R*)- and (*S*)-4′-hydroxy-flurbiprofen formation following co-incubation of (*R*)- and (*S*)-flurbiprofen as substrates in human liver samples

	K_m [μM]		V_{max} [pmol·min^{-1}·(mg protein)$^{-1}$]	
Sample	(*S*)-4′OHF*	(*R*)-4′OHF†	(*S*)-4′OHF	(*R*)-4′OHF
1	1.2	1.1	533	420
4	1.0	0.8	203	158

See "Data analysis" for explanation of data-fitting procedures. Liver samples used in this experiment correspond to the same No. 1 and No. 4 samples from Table 1.
* (*S*)-4′-Hydroxy-flurbiprofen.
† (*R*)-4′-Hydroxy-flurbiprofen.

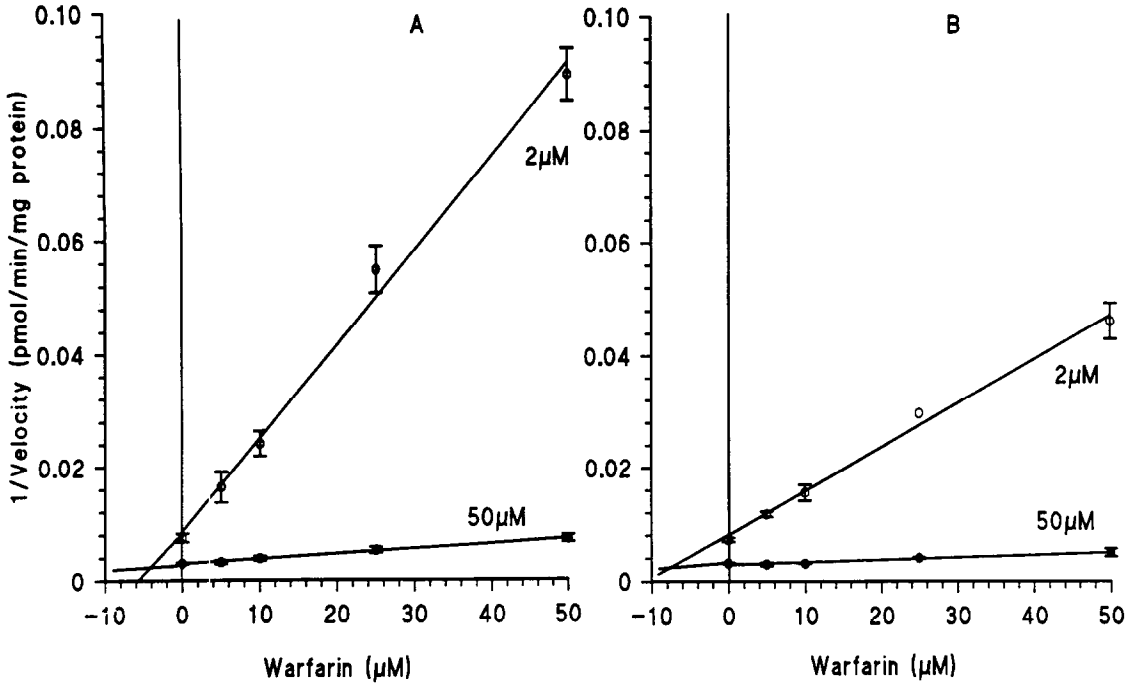

Fig. 4. Dixon plot representation of the inhibition of (*R*)-4′-hydroxy-flurbiprofen (panel A) and (*S*)-4′-hydroxyflurbiprofen (panel B) formation by *racemic*-warfarin. (*R*)- and (*S*)-Flurbiprofen at either 2 or 50 μM concentrations were incubated in the presence of warfarin concentrations ranging from 0 to 50 μM. The estimated K_i value for inhibition of (*R*)-4′-hydroxy-flurbiprofen formation was 2.2 ± 0.3 μM (SEM). The estimated K_i value for inhibition of (*S*)-4′-hydroxy-flurbiprofen formation was 4.7 ± 1.1 μM (SEM).

inhibitors of P450 forms or related forms. *Racemic*-warfarin, a putative P450 2C9 inhibitor [14], produced marked inhibition of (*R*)- and (*S*)-flurbiprofen 4′-hydroxylation with K_i values of 2.2 and 4.7 μM for inhibition of the two enantiomers, respectively (Fig. 4). Tolbutamide, which inhibits both P450 2C8 and P450 2C9 to varying degrees, also was studied as an inhibitor of this oxidative process [15]. Inhibition by tolbutamide was observed in the range appropriate for P450 2C9 [16] but with complicated kinetics that may involve a non-competitive component (data not shown). Erythromycin (an inhibitor of P450s in the P450 3A subfamily [17]) and α-naphthoflavone (an inhibitor of P450s in the P450 1A subfamily [18]) were also studied as potential inhibitors of (*R*)- and (*S*)-flurbiprofen 4′-hydroxylation. However, at concentrations of 0.5 and 5 μM, neither agent caused any marked inhibition of this oxidative process (data not shown).

To elucidate more conclusively the P450 forms involved in the 4′-hydroxylation of (*R*)- and (*S*)-flurbiprofen, we undertook studies using vaccinia virus-expressed P450s. Each of the flurbiprofen enantiomers was incubated with cells expressing either P450 1A2, 2C8, 2C9, 2C9R144C, 2E1 or 3A4 or cells infected with wild-type vaccinia virus. The P450 producing substantial 4′-hydroxlation of either (*R*)- or (*S*)-flurbiprofen was P450 2C9 and the variant 2C9R144C (Fig. 5). The rare alleles R144C variant produced a velocity roughly 1/4 that of P450 2C9. Similar results, although more pronounced, were found using (*S*)-warfarin in which V_{max} values were 17-fold greater than those of P450 2C9R144C [16]. The only other P450 demonstrating any activity was P450 1A2, but its turnover rate was 1/140th of that for P450 2C9R144C. Additionally, kinetic studies were performed with P450 2C9 cells for comparison to the K_m values obtained in human liver. The K_m values for formation of (*R*)- and (*S*)-4′-hydroxy-flurbiprofen were 6.6 and 4.3 μM, respectively (data not shown), being similar to those obtained in human liver samples (3.1 and 1.9 μM). It is also of note that no stereoselectivity was seen in the formation of either (*R*)- or (*S*)-4′-hydroxy-flurbiprofen with respect to turnover rate or V_{max} [31.0 and 31.4 pmol·min^{-1}· (pmol P450)$^{-1}$ for (*R*)- or (*S*)-4′-hydroxy-flurbiprofen, respectively (data not shown)], thus confirming that there is no stereoselectivity with respect to V_{max}.

DISCUSSION

NSAIDs, such as flurbiprofen, have been used widely in the clinic for several years. Furthermore, much has been published concerning their clinical efficacy and pharmacokinetics, in particular the metabolic inversion process that occurs with

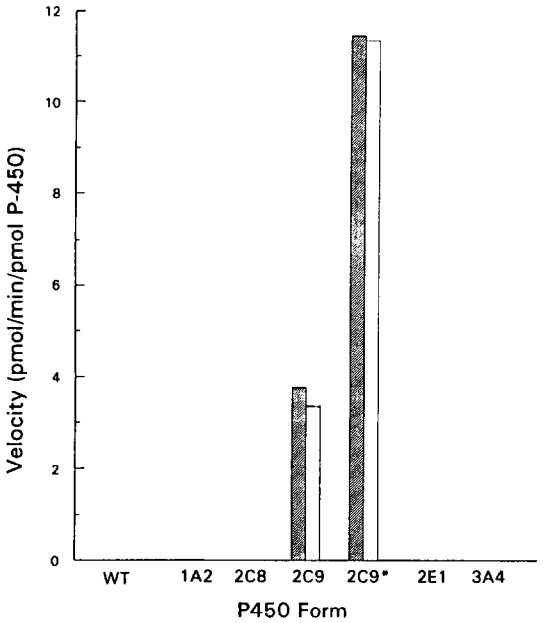

Fig. 5. Formation of either (*S*)-4′-hydroxy-flurbiprofen (hatched bars) or (*R*)-4′-hydroxy-flurbiprofen (open bars) by vaccinia virus-expressed P450s. Data labeled 2C9* = most common variant of P450 2C9. Data labeled 2C9 = R144C variant. The flurbiprofen substrate concentrations was 25 μM for both enantiomers in all incubations.

ibuprofen and fenoprofen. However, despite understanding the metabolic profile of NSAIDs like flurbiprofen, to date the specific P450 responsible for the oxidative biotransformation process is unknown. The study described herein has attempted to elucidate the stereoselectivity of one of the biotransformations of flurbiprofen (4′-hydroxylation), to determine whether interactions occur when both enantiomers are present, and to identify the specific P450 form responsible for the oxidative metabolism of this model compound.

Formation of 4′-hydroxy-flurbiprofen accounts for the overwhelming majority (86%) of the oxidative biotransformation of flurbiprofen [6], and *in vivo* studies of urinary excretion patterns indicate very little stereoselectivity in the formation of 4′-hydroxy-flurbiprofen [5]. Our studies indicate that only slight stereoselectivity may be present with respect to affinity of the flurbiprofen enantiomers for the oxidative enzyme, and no stereoselectivity exists with respect to the maximum velocity for the reaction (Table 1). Thus, these *in vivo* and *in vitro* results appear to be in concordance. Though a small portion (14%) of the metabolites excreted during *in vivo* studies are not accounted for here, these would have very little impact on the overall stereoselectivity of flurbiprofen biotransformation. Certainly, interpretation of the stereoselectivity of flurbiprofen oxidation is made simpler as flurbiprofen does not undergo metabolic (*R*)- to (*S*)-inversion of configuration as do other related NSAIDs such as ibuprofen and fenoprofen [19].

Enantiomeric interactions were observed when (*R*)- and (*S*)-flurbiprofen were employed simultaneously as substrates (Table 2, Fig. 3). These results would suggest that either the enantiomers were competing for the same enzyme or that one of the enantiomers binds to the enzyme but is not metabolized to any appreciable extent by the enzyme. Examples of both situations have been observed. Kunze *et al.* [20] have reported that the (*R*)-enantiomer of warfarin can inhibit (*S*)-warfarin 7-hydroxylation. It was later demonstrated that (*S*)-warfarin 7-hydroxylation was catalyzed predominantly by P450 2C9 and that (*R*)-warfarin was most likely binding to the active site but its turnover number was too low to be measured. Conversely, Kroemer *et al.* [21] have reported that (*S*)- and (*R*)-propafenone are both metabolized by P450 2D6 and competitively interact. Due to the enantiomeric differences in affinity for the P450 and the differing pharmacodynamic properties, a greater difference in pharmacodynamic outcomes is observed when the racemate is given than would be predicted based on the summation of the effects observed with the individual enantiomers [22]. In the case of flurbiprofen 4′-hydroxylation, it appears that the enantiomers are competing for the same P450 form (Fig. 5).

Chemical inhibition studies were used to ascertain the P450 forms responsible for flurbiprofen 4′-hydroxylation. That both *racemic*-warfarin and tolbutamide inhibited the 4′-hydroxylation of both (*R*)- and (*S*)-flurbiprofen would suggest that P450 2C9 and possibly P450 2C8 may be involved in flurbiprofen metabolism [15, 20]. The K_i values for warfarin (Fig. 4) are in agreement with the values obtained previously for warfarin metabolism [16]. It is unclear, however, why α-naphthoflavone did not inhibit this reaction as this compound has been reported recently to be an inhibitor of P450 2C9 as well as the P450 1A enzymes [23]. Since *racemic*-warfarin and tolbutamide can potentially inhibit P450 forms other than P450 2C8 and 2C9, these chemical inhibition studies were complemented with experiments utilizing cDNA expression of single P450 forms. These experiments confirmed that only P450 2C9, and not P450 2C8, was involved in this oxidation reaction.

Thus, it appears that the 4′-hydroxylation of flurbiprofen exhibits little stereoselectivity, particularly when both enantiomers are present, as appears clinically. Furthermore, it appears that of the P450 forms studied, only P450 2C9 was responsible for this oxidative reaction. However, further studies are needed to demonstrate conclusively whether P450 2C9 is the only isoform involved to any appreciable extent in catalyzing this reaction or whether related (e.g. P450 2C19) isoforms are involved.

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