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# Studies of Flurbiprofen 4′-Hydroxylation

## ADDITIONAL EVIDENCE SUGGESTING THE SOLE INVOLVEMENT OF CYTOCHROME P450 2C9

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**ABSTRACT.** Flurbiprofen, a non-steroidal anti-inflammatory drug (NSAID), is metabolized by both oxidation via the cytochrome P450 system and by glucuronidation. The major oxidative pathway in flurbiprofen metabolism is to a 4′-hydroxy metabolite, and recently we demonstrated that cytochrome P450 2C9 and its R144C variant were involved in this process (Tracy *et al.*, *Biochem Pharmacol* **49:** 1269–1275, 1995). Using complementary DNA (cDNA)-expressed cell systems, it has been demonstrated that at physiological concentrations of flurbiprofen there is a lack of involvement of P450s 1A2, 2C8, 2E1, and 3A4. In evaluating flurbiprofen as a potential probe for cytochrome P450 2C9, it is important to assess the involvement of additional P450s in this process. To this end, further studies were undertaken using specific inhibitors of P450 2C9 and P450 cDNA-expressed microsomes for P450 1A1, 2A6, 2B6, 2C19, and 2D6 to assess their potential involvement. We observed the inhibition of (*R*)- and (*S*)-flurbiprofen 4′-hydroxylation by an inhibitor of P450 2C9, sulfaphenazole ($K_i$ = 0.07 and 0.06 μM, respectively), and the NSAID piroxicam ($K_i$ = 10 and 7 μM, respectively). Furthermore, using microsomes from a lymphoblastoid cell line, we found that P450s 1A1, 2A6, 2B6, 2C19, and 2D6 were not involved in flurbiprofen hydroxylation at physiological concentrations of flurbiprofen. This finding is particularly important due to the sequence homology and potential substrate overlap of P450 2C9 and 2C19. These studies then provide additional evidence to suggest that P450 2C9 may be the only isoform involved to any substantial degree in flurbiprofen 4′-hydroxylation, and thus this reaction is useful as an *in vitro* probe for this particularly cytochrome P450 isoform and may be useful as an *in vivo* probe. BIOCHEM PHARMACOL 52;8:1305–1309, 1996.

**KEY WORDS.** flurbiprofen; cytochrome P450; human liver microsomes; vaccinia virus; cDNA expression

Flurbiprofen (racemic-2-[2-fluoro-4-biphenyl]propionic acid) belongs to the therapeutic class termed NSAIDs,¶ which are used in the treatment of pain or inflammation [1]. Administered as a racemic mixture, the flurbiprofen enantiomers are oxidized to form 4′-hydroxy-flurbiprofen and 3′4′-dihydroxy-flurbiprofen or further methylated to form 3′-hydroxy-4′methoxy-flurbiprofen [2, 3]. These metabolites as well as the parent compounds are also subject to conjugation by either glucuronidation or sulfation [2, 3]. With respect to oxidation, the formation of 4′-hydroxy-flurbiprofen accounts for 86% of the oxidative metabolites [4] and occurs via the P450 enzyme system.

We have demonstrated that this 4′-hydroxylation of both (*R*)- and (*S*)-flurbiprofen is carried out by P450 2C9 and its allelic variant P450 2C9R144C [5]. Furthermore, using a combination of putative specific inhibitors and P450 cDNA-expressed cells, we were able to demonstrate that P450s 1A2, 2C8, 2E1, and 3A4 are not involved in this hydroxylation. However, questions still remain concerning whether other P450s that play a substantial role in xenobiotic metabolism, most notably P450 2C19 but also 1A1, 2A6, 2B6, and 2D6, might also be involved in the 4′-hydroxylation of flurbiprofen.

Thus, to more completely assess the P450 isoforms involved in flurbiprofen 4′-hydroxylation, we extended our previous studies to assess whether the aforementioned isoforms (1A1, 2A6, 2B6, 2C19, and 2D6) might also be involved in this oxidative process. Additionally, we conducted inhibition studies using sulfaphenazole, an inhibitor of P450 2C9 [6], and piroxicam, another NSAID of a different chemical class that is purported to be metabolized by

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¶ *Abbreviations*: NSAID, non-steroidal anti-inflammatory drug; cDNA, complementary deoxyribonucleic acid; and CYP, cytochrome P450.

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P450 2C9 [7], to provide additional evidence for the involvement of P450 2C9 in the 4′-hydroxylation of the flurbiprofen enantiomers. The results of these studies are reported herein.

## MATERIALS AND METHODS

Ethyl acetate, acetonitrile, and potassium phosphate were obtained from the Fisher Co. (Pittsburgh, PA). Sulfaphenazole and piroxicam 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 for preparation of microsomes was obtained under protocols approved at the Medical College of Wisconsin. Based on chart review, none of the patients from whom tissues were obtained was taking any medications known to inhibit or induce the P450s. Microsomal samples were prepared according to established methods [8]. Protein content was measured by the method of Lowry *et al.* [9] and P450 content by the method of Omura and Sato [10]. Microsomal preparations from transfected human B-lymphoblastoid cell lines expressing human P450s 1A1, 2A6, 2B6, 2C19, and 2D6 or expressing only reductase were obtained from the Gentest Co. (Woburn, MA).

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 units glucose-6-phosphate dehydrogenase and 100 mM $K_2HPO_4$, pH 7.4, in a total volume of 200 μL and carried out for 20 min at 37°. 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 units of glucose-6-phosphate dehydrogenase was added, and the total volume was 400 μL. In experiments using CYP2A6, a 50 mM Tris buffer, pH 7.4, replaced the phosphate buffer, because the phosphate buffer inhibits the activity of P450 2A6 in this microsomal preparation (Gentest product information).

The measurement of 4′-hydroxy-flurbiprofen after incubation of either (*R*)- or (*S*)-flurbiprofen was carried out as described previously [5]. The effects of sulfaphenazole and piroxicam on the formation of 4′-hydroxy-flurbiprofen were evaluated by estimating the apparent inhibition constant ($K_i$) for the inhibitors according to the methods of Cleland [11].

## RESULTS

The 4′-hydroxylation of both (*R*)- and (*S*)-flurbiprofen was readily inhibited by addition of sulfaphenazole (Fig. 1). Sulfaphenazole exhibited a $K_i$ for the inhibition of (*S*)-4′-hydroxy-flurbiprofen formation of 0.06 μM, while the $K_i$ for the inhibition of (*R*)-4′-hydroxy-flurbiprofen formation was 0.07 μM. Piroxicam, an NSAID, was also tested as a potential inhibitor of 4′-hydroxy-flurbiprofen formation

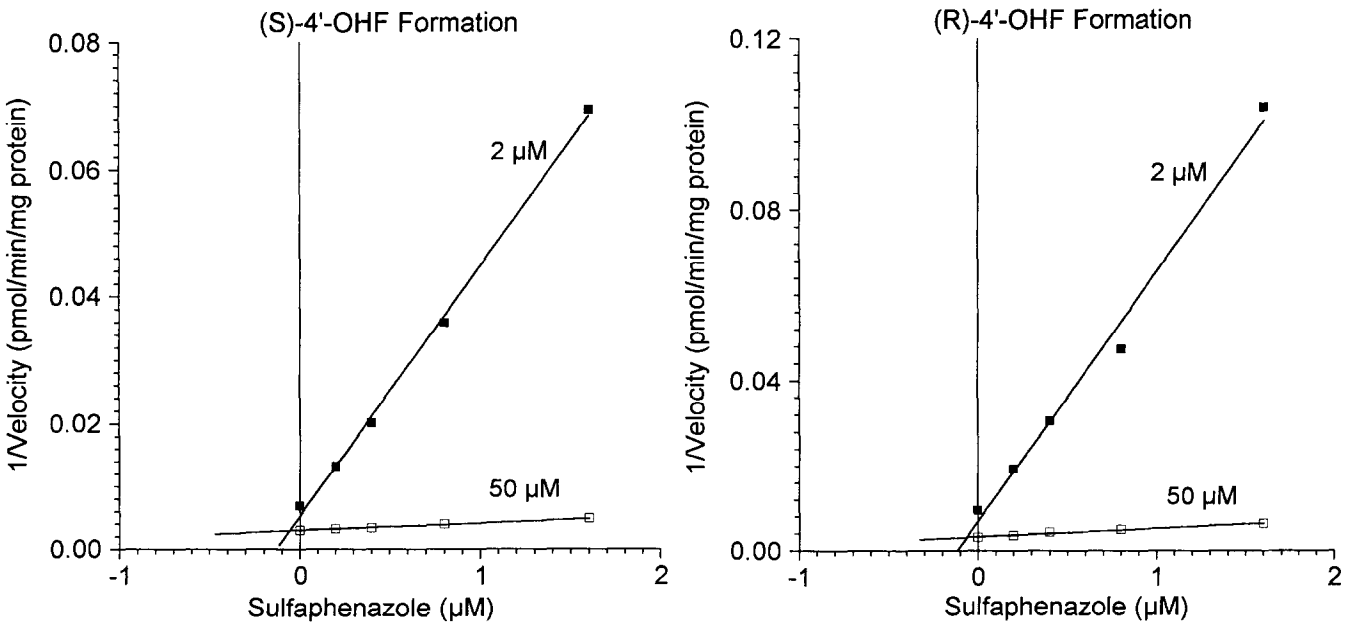


**FIG. 1. Dixon plot analysis showing inhibition of (*S*)-4′-hydroxy-flurbiprofen (left panel) and (*R*)-4′-hydroxy-flurbiprofen formation (right panel) by sulfaphenazole in human liver microsomes. Closed squares represent 2 μM flurbiprofen substrate concentration, and open squares represent 50 μM flurbiprofen substrate concentration. Solid lines indicate best linear regression fit of the data points. Estimated $K_i$ values for inhibition of (*S*)- and (*R*)-4′-hydroxy-flurbiprofen formation by sulfaphenazole were 0.06 and 0.07 μM, respectively.**

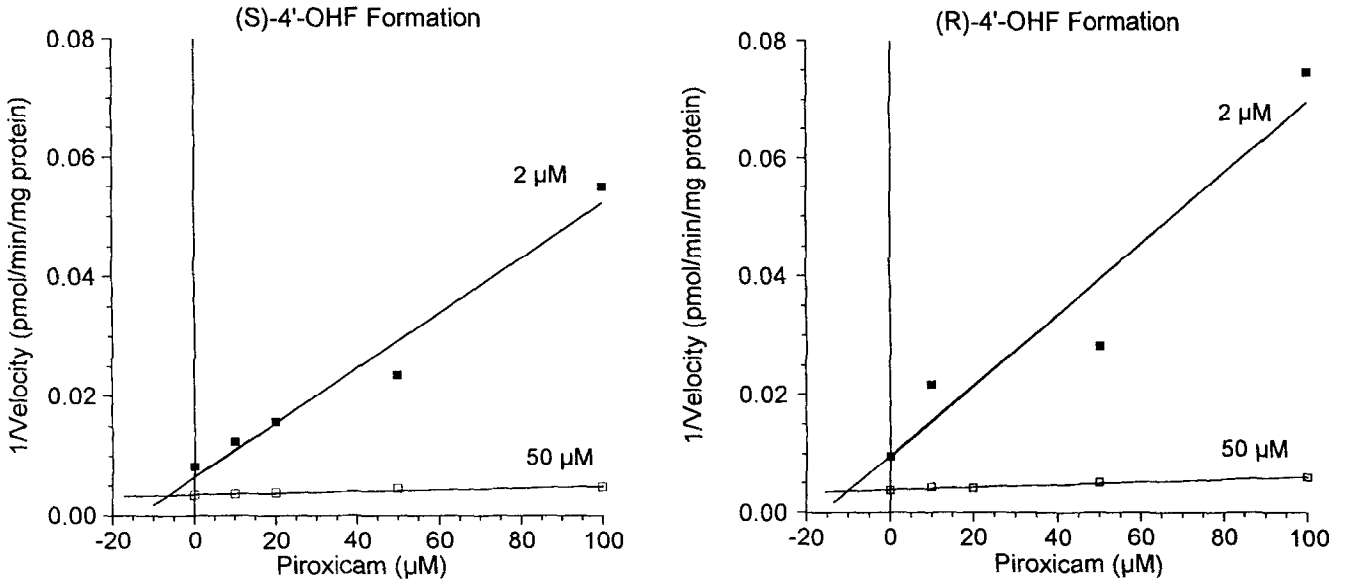


**FIG. 2. Dixon plot analysis showing inhibition of (S)-4'-hydroxy-flurbiprofen (left panel) and (R)-4'-hydroxy-flurbiprofen formation (right panel) by piroxicam in human liver microsomes. Closed squares represent 2 μM flurbiprofen substrate concentration, and open squares represent 50 μM flurbiprofen substrate concentration. Solid lines indicate best linear regression fit of the data points. Estimated $K_i$ values for inhibition of (S)- and (R)-4'-hydroxy-flurbiprofen formation by piroxicam were 7 and 10 μM, respectively.**

(Fig. 2). Piroxicam also inhibited hydroxylation of both (S)-($K_i$ = 7 μM) and (R)-flurbiprofen ($K_i$ = 10 μM), though with higher $K_i$ values than observed for sulfaphenazole.

The involvement of additional P450s, other than P450 2C9, was assessed by incubating both (R)- and (S)-flurbiprofen in the presence of microsomes from lymphoblastoid cell lines expressing P450s 1A1, 2A6, 2B6, 2C19, and 2D6. None of these P450 isoforms (1A1, 2A6, 2B6, 2C19, or 2D6) formed 4'-hydroxy-flurbiprofen under the conditions studied. These data have been combined with data previously generated [5] to give a complete picture of the P450 isoforms studied to date with respect to flurbiprofen 4'-hydroxylation (Fig. 3).

## DISCUSSION

Much effort is being expended to identify compounds that can be given to patients in an effort to phenotype the individual with respect to the P450 isoforms and other metabolizing enzymes. Prior to administration to humans, compounds to be used as probes for specific enzymes must be evaluated using *in vitro* techniques to determine the specific isoforms involved in the metabolism of a compound. In this regard, we have embarked on a series of *in vitro* studies to ascertain the P450 isoform(s) involved in the 4'-hydroxylation of flurbiprofen. Previously, we have shown that P450 2C9 and its R144C allelic variant are involved in 4'-hydroxy-flurbiprofen formation [5]. Additionally, through the use of inhibitors and cDNA-expressed cell systems we had demonstrated the lack of involvement of P450s 1A2, 2C8, 2E1, and 3A4 [5]. Extension of those studies reported herein demonstrate the lack of involvement of P450s 1A1, 2A6, 2B6, 2C19, and 2D6. Inhibition by the P450 2C9 inhibitor sulfaphenazole as well as the proposed P450 2C9 substrate piroxicam further confirmed the involvement of P450 2C9 (though the selectivity of sulfaphenazole for P450 2C9 has not been proven conclusively). These additional studies are particularly important in light of the similar homology of P450 2C19 to P450 2C9 [12] and their potential cross-reactivity of substrates. For example, P450 2C19 is responsible for the 4'-hydroxylation of (S)-mephenytoin but (R)-mephenytoin is also metabolized by P450 2C9 [13, 14]. Previous studies demonstrating the ability of racemic-mephenytoin to inhibit the 5'-hydroxylation of piroxicam and tenoxicam [7] left open the possibility of the involvement of P450 2C19 in NSAID metabolism. Thus, the demonstrated lack of participation of P450 2C19 in flurbiprofen 4'-hydroxylation resolves this heretofore unanswered question concerning the potential involvement of P450 2C19.

During the design of *in vitro* metabolism studies, consideration of the relevance of incubation substrate concentrations must be considered as it has been suggested recently that depending on the substrate concentration used, one may be led inadvertently to proposed involvement of a particular P450 isoform in the metabolism of a compound that may not be involved at actual physiological concentrations (for example, chlorzoxazone metabolism) [15–17]. We chose a substrate concentration (12.5 μM) to screen the metabolism of flurbiprofen that was well above the $K_m$ values we observed in both human liver microsomes [1.9 and 3.1 μM for (S)- and (R)-flurbiprofen, respectively] and

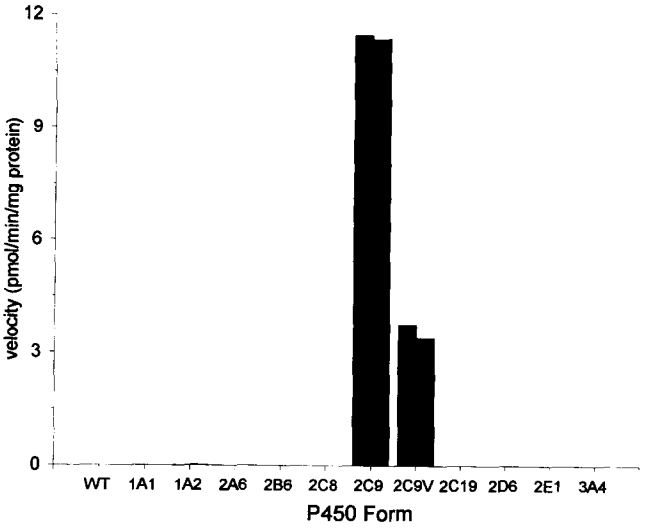


**FIG. 3. Formation of either (*S*)-4′-hydroxy-flurbiprofen (gray bars) or (*R*)-4′-hydroxy-flurbiprofen (black bars) by vaccinia virus expressed P450s. Data labeled 2C9v = R144C variant. Flurbiprofen substrate concentrations were 12.5 μM for both enantiomers in all incubations. The only other isoform demonstrating flurbiprofen 4′-hydroxylase activity different from control, apart from P450 2C9 and its variant, was P450 1A2 [0.0126 and 0.0224 pmol/min/pmol P450 for (*S*)- and (*R*)-4′-hydroxy-flurbiprofen formation, respectively] making the activity of this isoform roughly 500 times lower than that of P450 2C9. This activity detected for P450 1A2 was just slightly above the detection limit for the assay. Data for P450s 1A2, 2C8, 2C9, 2C9v, 2E1, and 3A4 are adapted from data originally published in *Biochem Pharmacol*, Tracy *et al.*, "Role of cytochrome P450 2C9 and an allelic variant in the 4′-hydroxylation of (*R*)- and (*S*)-flurbiprofen," 49: 1269–1275, 1995, cited with permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, U.K.**

cDNA-expressed cells [4.3 and 6.6 μM for (*S*)- and (*R*)-flurbiprofen, respectively] [5] and was at or near those concentrations producing maximum velocity but well within those concentrations typically seen in patients [18]. Thus, this concentration (12.5 μM) should be appropriate for *in vitro–in vivo* correlations of flurbiprofen metabolism, and the findings lend additional support to the observation that P450 2C9 plays a major, if not exclusive role in the 4′-hydroxylation of (*R*)- and (*S*)-flurbiprofen.

Based on the evidence accumulated to date, flurbiprofen 4′-hydroxylation serves well as an *in vitro* probe for cytochrome P450 2C9. Due to its high turnover rate and the excellent sensitivity of the assay, the formation of 4′-hydroxy-flurbiprofen can be measured easily at very low amounts (~2 pmol injected on the column). Thus, one can perform microsomal incubations on a very small scale using only 20 μg of microsomal protein per 200 μL incubation. Furthermore, these assays can be performed easily at concentrations expected to be encountered physiologically following oral administration. The next challenge is to determine the appropriateness of flurbiprofen as an *in vivo* probe for cytochrome P450 2C9. One potential obstacle is that flurbiprofen is marketed as a racemate, and thus total 4′-hydroxy-flurbiprofen concentrations in plasma or amounts excreted renally may not reflect P450 2C9 activity (due to small stereoselectivity differences in the rate of elimination in subjects [19] and thus necessitates a stereoselective assay or administration of a single enantiomer. Studies are currently underway to assess the appropriateness of flurbiprofen and whether administration of the racemate will be adequate.

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