ABSTRACT:

The forms of cytochrome P450 involved in the stereoselective S-oxidation of flosequinan ([±]-7-fluoro-1-methyl-3-methylsulfonyl-4-quinolone) were investigated in vitro using liver microsomes from rats and humans. Rat liver microsomes supplemented with NADPH catalyzed the four different S-oxidations, which were from flosequinan sulfide (FS; 7-fluoro-1-methyl-3-methylthio-4-quinolone) to R(+)- and S(−)-flosequinan (R-FSO and S-FSO, respectively) and from R-FSO and S-FSO to flosequinan sulfone (FSO2; 7-fluoro-1-methyl-3-methylsulfonyl-4-quinolone). The activities of all the S-oxidases in liver microsomes from male rats were higher than those from female rats. The activities of the S-oxidases measured at a high substrate concentration (1 mM) were induced by treatment of rats with 3-methylcholanthrene and dexamethasone. Treatment of rats with 3-methylcholanthrene also induced the activities, but only at a low substrate concentration (50 μM), except for the S-oxidase catalyzing the reaction from FS to R-FSO. Enzymes induced by clofibrate and ethanol were not involved in the oxidations at a low substrate concentration. The activities of S-oxidases were correlated with the contents of cytochrome P450 (CYP)3A enzymes. Anti-CYP3A2 antisera inhibited the activities of the S-oxidases catalyzing the reactions from FS to R-FSO (40%) and to S-FSO (60%) at the high substrate concentration and inhibited the activities of the S-oxidases, thus catalyzing reactions from R-FSO and S-FSO to FSO2 (70%) at both high and low substrate concentrations. These results suggest that CYP3A is the major enzyme involved in all S-oxidation pathways in flosequinan metabolism in rats. On the other hand, except for the S-oxidation of FS to R-FSO, the rates of the other three S-oxidations by liver microsomes from 30 individual humans correlated highly with each other, suggesting that the same enzyme would be involved in the three S-oxidations. Anti-CYP3A2 antisera inhibited the activities of all the S-oxidases in human liver microsomes ranging from 40 to 80%, suggesting that CYP3A is also involved in all of the S-oxidations of flosequinan in humans.

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1 Abbreviations used are: R-FSO, R(+)-flosequinan; S-FSO, S(−)-flosequinan; FSO2, flosequinan sulfone; FS, flosequinan sulfide; P450, cytochrome P450; FMO, flavin-containing monoxygenase; FS, flosequinan; PB, phenobarbital; 3-MC, 3-methylcholanthrene; DEX, dexamethasone; CF, clofibrate; EtOH, ethanol; GS, generating system; CYP, cytochrome P450.

**Fig. 1. Proposed metabolic pathways and enzymes involved in the metabolism of flosequinan in rats.**

Consist of a number of isozymes (7, 8). The respective involvement of P450 and FMO in flosequinan metabolism was investigated in rats.\(^2\) The in vitro inhibition of S-oxidase activities by anti-P450 antibody, inhibitors, and antibody to P450 reductase suggested that P450 in rat liver microsomes catalyzed the four different S-oxidations in flosequinan metabolism. FMO catalyzed only the stereoselective S-oxidation...
The typical reaction mixture (200 μl) for the assay using rat liver microsomes consisted of 0.1 M Na\(^+\),K\(^+\)-phosphate buffer (pH 7.4), 0.1 mM EDTA, and 0.05 or 1 mM substrate and microsomes (0.5–1 mg protein/ml). After preincubation for 3 min at 37°C, the reaction was initiated by the addition of an NADPH-GS (5.0 mM glucose-6-phosphate, 0.5 mM NADPH\(^+\), 1 unit/ml glu-
TABLE 2

<table>
<thead>
<tr>
<th>Inducer</th>
<th>FS → S-FSO</th>
<th>R-FSO → S-FSO</th>
<th>R-FSO → FSO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Km₁</td>
<td>Vmax₁</td>
<td>Km₂</td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>mmol/min/mg protein</td>
<td>μM</td>
</tr>
<tr>
<td>3-MC</td>
<td>27</td>
<td>220</td>
<td>16</td>
</tr>
<tr>
<td>PB</td>
<td>220</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>DEX</td>
<td>400</td>
<td>16</td>
<td>40</td>
</tr>
</tbody>
</table>

Kinetic Analysis. Enzyme kinetic parameters (Km, Vmax, Vmax/Km) were estimated by the linear regression analysis of simple Michaelis-Menten kinetics, also the nonlinear least-squares regression analysis of Eadie-Hofstee plots. Substrate concentrations varied from 0.01 to 1 mM.

Immunoinhibition of Flosequinan Metabolism by Antisera. Antisera to the purified P450s were raised in rabbits and goats as described previously (14, 15). The ability of antisera to inhibit the formation of flosequinan metabolites was measured in the presence of a desired amount of the antisera indicated in the figures. The mixture of antisera and microsomes was preincubated for 60 min at room temperature before incubations. All further reaction conditions were the same as described.

Immunoblot Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot were performed according to the methods of Laemmli (16) and Guengerich et al., (17) with a 7.5% polyacrylamide gel. Immunodetection was performed with the biotinylated antibody-avidin-peroxidase system (Vector Laboratories, Burlingame, CA). 4-Chloro-1-naphthol was used as a substrate for peroxidase. The content of each form of P450 and FMO was determined by measuring the immunostained bands with a densitometer (Quick Scan R&D, Helena Laboratory, Beaumont, TX).

Results

To investigate the involvement of P450 isozymes in the four different S-oxidation pathways of flosequinan in rats, the activities of the S-oxidases in liver microsomes from untreated male and female rats and from male rats treated with an inducer were compared. The S-oxidase activities measured at substrate concentrations of 50 μM and 1 mM are summarized in fig. 2. The rates of S-oxidation from FS to R-FSO and S-FSO were >10 times higher than those from R-FSO and S-FSO to FSO₂ in untreated male rats at the low substrate concentration. The rates of all S-oxidations seen in untreated female rats were lower than those of untreated male rats, suggesting the involvement of the male-specific or predominant enzymes in these reactions. The effects of inducers (3-MC, PB, EtOH, DEX, and CF) known to elevate a certain subfamily of P450 contents were investigated (18). The activity of enzymes catalyzing the S-oxidation from FS to R-FSO at the low concentration of FS was not increased by treatment of rats with inducers used in the present study. At the high concentration of FS, microsomes from rats treated with PB and DEX showed a higher S-oxidase activity. On the other hand, 3-MC, PB, and DEX treatments increased all other S-oxidase activities at the low substrate concentration. The CF treatment increased all four different S-oxidase activities measured at only the high substrate concentration.
The rates of four different S-oxidations in liver microsomes from EtOH-treated rats did not differ from untreated male rats measured at both substrate concentrations, indicating that enzymes induced by EtOH are not involved in the flosequinan metabolism.

The analysis of correlations between the four different S-oxidase activities in liver microsomes from individual untreated male and female rats and rats treated with inducers is shown in table 1. The rates of S-oxidations from FS to R-FSO and from FS to S-FO at the low FS concentration did not correlate with the rates at the high FS concentration ($r \approx 0.53$), indicating that different enzymes were involved in the metabolism of flosequinan.

Table 1: Correlation between the S-oxidase activities in flosequinan metabolism and the content of CYP subfamily or FMO in rat liver microsomes

<table>
<thead>
<tr>
<th>P450 and FMO</th>
<th>FS → R-FO</th>
<th>FS → S-FO</th>
<th>R-FO → FSO2</th>
<th>S-FO → FSO2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 μM</td>
<td>1 mM</td>
<td>50 μM</td>
<td>1 mM</td>
</tr>
<tr>
<td></td>
<td>[correlation coefficient (r)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.26</td>
<td>0.19</td>
<td>0.68</td>
<td>0.03</td>
</tr>
<tr>
<td>CYP2B1</td>
<td>0.15</td>
<td>0.36</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>CYP2C6</td>
<td>0.05</td>
<td>0.24</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>0.55</td>
<td>0.05</td>
<td>0.23</td>
<td>0.05</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>0.04</td>
<td>−0.17</td>
<td>−0.22</td>
<td>−0.21</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>0.21</td>
<td>0.87</td>
<td>0.25</td>
<td>0.85</td>
</tr>
<tr>
<td>CYP4A1</td>
<td>−0.29</td>
<td>0.08</td>
<td>−0.22</td>
<td>0.08</td>
</tr>
<tr>
<td>FMO</td>
<td>0.55</td>
<td>0.19</td>
<td>0.23</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Fig. 3. Immunoinhibition by anti-P450s sera of the S-oxidase activities in flosequinan metabolism by microsomes from untreated male rats.

Results are presented as a remaining activity relative to control samples without antiserum. S-oxidase activities were determined as described in the text at substrate concentrations of 50 μM (A) or 1 mM (B). Symbols represent preimmune sera (○), anti-CYP1A2 (▲), anti-CYP2C11 (●), and anti-CYP3A2 (○) sera. prot., protein; Ms, microsomes.
TABLE 4  
Kinetic parameters for S-oxidations in human liver microsomes

<table>
<thead>
<tr>
<th>Human Liver</th>
<th>S-FOX → FSO 2</th>
<th>R-FOX → FSO 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (µM)</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/mg protein)</td>
</tr>
<tr>
<td>HLA</td>
<td>2.40</td>
<td>38</td>
</tr>
<tr>
<td>HLB</td>
<td>4.40</td>
<td>96</td>
</tr>
<tr>
<td>HLC</td>
<td>13.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Mean</td>
<td>17.00</td>
<td>1.70</td>
</tr>
<tr>
<td>SD</td>
<td>0.40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

For S-oxidations from FS to R-FSO and S-FSO, the low substrate concentration was 50 µM and 1 mM, respectively. On the other hand, the kinetic plots for S-oxidations from FS to R-FSO and S-FSO gave monophasic kinetic behavior. Although treatment with 3-MC caused essentially no changes in the K<sub>m</sub> and V<sub>max</sub> values for the S-oxidation from FS to R-FSO, the treatment decreased the K<sub>m</sub> values for the other three S-oxidations, and increased the V<sub>max</sub> values of a high-affinity component in the S-oxidation from FS to S-FSO and from R-FSO to FSO<sub>2</sub>. The S-oxidations from FS to R-FSO and S-FSO by liver microsomes from rats treated with PB and DEX gave apparently monophasic kinetic pattern. The K<sub>m</sub> values for the S-oxidations from FS to R-FSO and S-FSO by liver microsomes from rats treated with PB and DEX were between the K<sub>m</sub> values for the high- and low-affinity components seen with liver microsomes from untreated rats. On the other hand, PB and DEX treatment caused an increase of the V<sub>max</sub> values of the S-oxidations from R-FSO and S-FSO to FSO<sub>2</sub>, without changing the K<sub>m</sub> values.

To clarify the enzyme(s) responsible for the S-oxidations, the activities of the S-oxidase and the contents of each investigated form of P450 and FMO were correlated (table 3). All of the S-oxidase activities at the high substrate concentration correlated only with the contents of CYP3A. The activities of the S-oxidase at the low concentration of FS to R-FSO and S-FSO showed no apparent correlation with the content of CYP3A. The activities of the S-oxidase to produce FSO<sub>2</sub>, from R-FSO and S-FSO at the low substrate concentration correlated with the content of CYP3A protein.

To confirm the involvement of a P450 isozyme in the S-oxidations in liver microsomes from untreated rats, the effects of antibodies to rat CYP1A2, CYP2C11, and CYP3A2 on the activities of S-oxidases were examined at two substrate concentrations (50 µM and 1 mM) (fig. 3). Antibodies to CYP2B1, CYP2C6, CYP2E1, and CYP4A1 did not inhibit any of the S-oxidation reactions (data not shown). The activity of the S-oxidase involved in the reaction from FS to R-FSO was not inhibited by these antibodies. Although antibodies to CYP1A2, CYP2C11, and CYP3A2 inhibited the activity of S-oxidase from FS to S-FSO at the low concentration of the substrate, the inhibitions were <40%. At the high substrate concentration, the inhibition of the S-oxidase activity by anti-CYP3A2 from FS to S-FSO became apparent and inhibition reached 60%. Antibodies to CYP3A2 also inhibited >70% of the activities of S-oxidases from R-FSO and S-FSO to FSO<sub>2</sub> at both low and high substrate concentrations.
To find out whether the S-oxidations of flosequinan were catalyzed by the corresponding form of P450 in humans, we investigated the activities of S-oxidases in human liver microsomes, following similar methods using rat liver microsomes. Although the S-oxidation reactions from rac-FSO to FSO₂ in human liver microsomes were reported, no information was available on the stereoselectivity of these S-oxidation reactions. It was confirmed that human liver microsomes supplemented with NADPH also catalyzed the four stereoselective S-oxidations. We calculated Michaelis-Menten kinetic parameters for the S-oxidation of flosequinan in liver microsomes from three individual humans (table 4). Because the kinetic plots of all S-oxidations gave monophasic kinetics, each reaction was then tentatively assumed to be catalyzed by one enzyme. The $K_M$ and $V_{\text{max}}$ values for the S-oxidase catalyzing the reaction from FS to R-FSO were higher than those seen in the S-oxidase from FS to S-FSO, respectively. The $K_M$, $V_{\text{max}}$, $V_{\text{max}}/K_M$ values of the S-oxidase from R-FSO to FSO₂ were similar to those from S-FSO to FSO₂. Thus, the $V_{\text{max}}/K_M$ values for the S-oxidations from FS to R-FSO and S-FSO were 10 times higher than those from R-FSO and S-FSO to FSO₂.

The activities of the S-oxidases in liver microsomes from 30 individual humans were measured, and correlations among the activities were determined (fig. 4). The rates of three of the four S-oxidation reactions, with the S-oxidation from FS to R-FSO as the exception, showed high correlation ($r \geq 0.96$) with each other, indicating that the same enzyme(s) would be involved in the three S-oxidation reactions. In addition, the activities of S-oxidase catalyzing the reaction from FS to R-FSO also showed some correlations ($r \approx 0.63$) with the activities of the other S-oxidases, suggesting that a portion of the S-oxidation from FS to R-FSO might be catalyzed by the same enzyme(s) catalyzing the other three S-oxidations.

To clarify the enzyme(s) involved in the S-oxidations in human liver microsomes, immunoinhibition of the S-oxidases in liver microsomes from three individual humans by antibodies to rat P450 was performed (fig. 5). Antibodies to rat CYP3A2 inhibited 40 to 80% of the enzyme activities for four different S-oxidations, indicating that CYP3A in human liver microsomes was the major enzyme responsible for all S-oxidations.

**Discussion**

The enantiomers of flosequinan are oxidized to the sulfone as a major metabolite and reduced to the sulfide as a minor metabolite (3, 20). The sulfide form is oxidized to the parent enantiomers of flosequinan (fig. 1) (13). These are thought to be major metabolite pathways known so far. The reduction of the sulfoxide group may occur in vivo in tissues containing thioredoxin-linked enzymes (21).
and aldehyde oxidase (22) or intestinal bacteria (23). In the case of flosequinan, the reduction of a sulfoxide group was mainly catalyzed by intestinal bacteria. The reduction of $R$-FSO occurred more rapidly than that of $S$-FSO by intestinal content from rats (13).

It is known that the $S$-oxidation of drugs is catalyzed by P450 and FMO. We previously reported that P450 catalyzed all of four different $S$-oxidations, whereas FMO catalyzed only the $S$-oxidation from FS to $R$-FSO in the metabolism of flosequinan using rat liver microsomes (3). In the present study, we focused on the involvement of P450 isozymes in the stereoselective $S$-oxidation pathways of flosequinan in vitro in rats and humans.

It is known that CYP2C11, CYP2C13, and CYP3A2 are specific or predominant isozymes in male rats (24). Thus, it is suggested that CYP2C and/or CYP3A would mediate all $S$-oxidation pathways. 3-MC, EtOH, and DEX are typical inducers of CYP1A, CYP2E, and CYP3A, respectively (25–27). PB is a typical inducer of CYP2B and also a strong inducer of CYP3A (28). CF is a typical inducer of CYP4A and CYP2B (29). In the present study, the contents of P450 isozymes determined by Western blot analysis in liver microsomes from rats treated with the inducer were consistent with those reports.

It is suggested that CYP2E1 was not involved in these $S$-oxidations, and that three $S$-oxidations, except for the reaction from FS to R-FSO, were catalyzed by CYP1A induced by 3-MC and CYP3A induced by PB and DEX. CYP2B and/or CYP4A induced by CF may catalyze above three $S$-oxidations at only the high substrate concentration.

CYP3A and CYP2C are major forms, whereas CYP1A, CYP2B, and CYP4A are minor forms (28, 30) in untreated male rats. Therefore, the participation of these isoforms in the $S$-oxidation reactions would be minor in untreated male rats.

The $S$-oxidation pathway from FS to R-FSO was catalyzed by FMO as a high-affinity enzyme and by P450 as a low-affinity enzyme using rat liver microsomes. Recombinant rat FMO1A1 catalyzed the $S$-oxidation reaction from FS to R-FSO (9). Immunoinhibition study showed that CYP3A was not mainly involved in the $S$-oxidation from FS to R-FSO, probably because FMO is the major enzyme responsible for this $S$-oxidation pathway.

Previously, we reported that the pharmacokinetic parameters of $R$-FSO and $S$-FSO in male rats were similar after an intravenous administration of flosequinan (3), whereas the plasma concentrations of $R$-FSO were ~5 times higher than those of $S$-FSO after an oral administration of FS in male rats (13). In the present in vitro kinetic study, we showed that $V_{max}/K_M$ values for $S$-oxidation from R-FSO to FSO$_2$ and from $S$-FSO and FSO$_2$ were similar using untreated male rats, and that a $V_{max}/K_M$ value for $S$-oxidation from FS to R-FSO was >10 times higher than that from FS to S-FSO. These results can be well explained using the aforementioned in vivo pharmacokinetic behavior. An emphasis should be made that the kinetic study of the drug metabolism in vitro by using liver microsomes may be a useful approach for extrapolation of an in vivo drug disposition.

There is no report available on the chiral inversion of flosequinan
in humans \textit{in vivo}. However, the chiral inversion can be predicted by \textit{in vitro} studies on sulfoxide reduction and the S-oxidation reactions. Sulfoxide reduction is mainly catalyzed by intestinal bacteria (13). The extent of chiral inversion from S-FSO to R-FSO was greater than the reverse inversion in rat (3). This was estimated by the fact that the disproportional chiral inversion was caused by predominant stereoselective S-oxidation from FS to R-FSO in rats. It was confirmed in the present study that FS was oxidized to R-FSO and S-FSO \textit{in vitro} using human liver microsomes. Therefore, the chiral inversion would occur in humans \textit{in vivo}.

Although the plasma level of S-FSO was higher than that of R-FSO after oral administration, there are no differences in the $V_{max}/K_m$ value of the two stereoselective S-oxidations, respectively, in human liver microsomes. Therefore, it seemed possible to assume that the cause of the different pharmacokinetics of flosequinan enantiomers was not only the stereoselective S-oxidation, but also other events, such as a stereoselective sulfoxide reduction, absorption, excretion, etc.

Although the present study indicates that the CYP3A family is involved in all the S-oxidations in flosequinan metabolism in humans, it remains unclear which specific isozyme in the CYP3A family is responsible for the oxidations. It has been reported that several isozymes (such as CYP3A3, CYP3A4, CYP3A5, and CYP3A7) are present in human liver microsomes (31, 32). The fact that flosequinan was metabolized mainly by CYP3A suggests that flosequinan may be more rapidly eliminated in humans when an inducer of CYP3A (e.g. barbiturates (33)) is repeatedly administered to patients, and more slowly eliminated when an inhibitor such as gestodene, erythromycin, and other antibiotics (34, 35) is given simultaneously with flosequinan.

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