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Stereoselective *S*-oxidation and reduction of flosequinan in rat

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1. The stereoselective *S*-oxidation and reduction pathways of flosequinan [(±)-7-fluoro-1-methyl-3-methylsulphinyl-4-quinolone] in rat were investigated *in vitro*.

2. Cytosol from both the liver and kidney catalysed the reduction of *R*(+)-flosequinan (R-FSO) and *S*(−)-flosequinan (S-FSO) to flosequinan sulphide (FS, 7-fluoro-1-methyl-3-methylthio-4-quinolone). Flosequinan sulphone (FSO_2, 7-fluoro-1-methyl-3-methylsulphonyl-4-quinolone) was not reduced to R-FSO or S-FSO.

3. Liver microsomes catalysed four different *S*-oxidation pathways in the presence of NADPH, namely oxidation of FS to R-FSO and S-FSO and from R-FSO and S-FSO to FSO_2. The formation of R-FSO and S-FSO from FS each exhibited a biphasic kinetic pattern, indicating that at least two distinct enzymes were involved. The pathway from FS to R-FSO appeared mainly catalysed by flavin-containing monooxygenases (FMO).

4. *S*-oxidation of FS to R-FSO was more rapid than that of FS to S-FSO. *S*-oxidation of FS to either R-FSO or S-FSO in liver microsomes was more rapid than that of either R-FSO or S-FSO to FSO_2.

5. Microsomes from both the kidney and lung catalysed the stereoselective *S*-oxidation of FS to R-FSO, and FMO was likely to have participated in these reactions.

Introduction

Flosequinan [*rac*-FSO, (±)-7-fluoro-1-methyl-3-methylsulphinyl-4-quinolone] is a peripheral vasodilator with therapeutic effects on both arterial and venous vascular beds (Cowley *et al*. 1984, Yates 1991). The compound has a chiral sulphur atom, resulting in two stereo-isomers, (+)-(*R*)- and (−)-(*S*)-flosequinan (R-FSO and S-FSO). The stereoselective pharmacokinetics in rat have been investigated and reported by Kashiyama *et al*. (1994b). Each enantiomer showed a different *in vivo* pharmacokinetic behaviour. Chiral interconversion appeared to occur through the formation of flosequinan sulphide (FS, 7-fluoro-1-methyl-3-methylthio-4-quinolone), since R-FSO and S-FSO were produced when FS was intravenously administered to rat. The proposed *in vivo* metabolic pathways are shown in figure 1. While FS is formed by the reduction of both enantiomers, R-FSO and S-FSO are formed by the *S*-oxidation of FS. When each enantiomer is administered *in vivo*, the main metabolite in both cases is flosequinan sulphone (FSO_2, 7-fluoro-1-methyl-3-methylsulphonyl-4-quinolone). FSO_2 seems to be the terminal metabolite, since

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only FSO_2 was observed when FSO_2 was intravenously administered to rat. Therefore, there are four *S*-oxidation and two reduction pathways for flosequinan metabolism in rat *in vivo*.

Sulphur-containing drugs are known to be metabolized via *S*-oxidation and reduction pathways. The oxidation of sulphur compounds is catalysed almost exclusively by cytochrome P450-dependent monooxygenases (P450) and flavin-containing monooxygenases (FMO) in the liver. P450s are localized in many tissues of mammals and consist of many isozymes (Nelson *et al*. 1996). P450s play an essential role in the oxidative metabolism of endogenous compounds and of foreign chemicals, including drugs (Kulkarni and Hodgson 1980). FMOs catalyse the NADPH-dependent oxidative metabolism of sulphur-, nitrogen- and phosphorous-containing drugs (Ziegler 1980, 1988). Distinct FMO proteins and cDNA have been purified and isolated from mouse, rat, rabbit, guinea pig, pig and human (Lawton and Philpot 1993, Ziegler 1993). Northern and Western blot analyses have indicated that FMO are mainly expressed in the liver, lung and kidney (Tynes and Philpot 1987, Itoh *et al*. 1993). Despite detailed studies on the oxidation of drugs, the mechanisms responsible for the reduction of S-oxides have been reported for only a few chemicals. For example, it has been reported that thioredoxin-linked enzyme systems and/or aldehyde oxidase are capable of reducing sulphoxide-containing compounds in the liver and kidney (Aymard *et al*. 1979, Fukazawa *et al*. 1987, Anders *et al*. 1981, Yoshihara and Tatsumi 1985).

To clarify the mechanisms responsible for the chiral interconversion of flosequinan, the present study was conducted to investigate which enzymes are involved in the stereoselective *S*-oxidation and reduction of flosequinan *in vitro*.

Materials and methods

Chemicals

rac-FSO, FS and FSO_2 were supplied by Boots Co. (Nottingham, UK). R-FSO and S-FSO were prepared (Morita *et al*. 1994) by the Tokushima Research Institute of Otsuka Pharmaceutical Co. (Tokushima, Japan). The optical purity of R-FSO and S-FSO was >99% as determined by hplc. Other chemicals were obtained from the following sources: glucose oxidase, dithiothreitol (DTT) and *n*-octylamine from Wako Pure Chemical Industries (Osaka, Japan), 2-hydroxypyrimidine (2-OH-PM) and 1-(1-naphthyl)-2-thiourea (naphthylthiourea) from Tokyo Chemical Industry (Tokyo, Japan), SKF-525A hydrochloride from Funakoshi (Tokyo, Japan), $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase from Oriental Yeast (Tokyo, Japan). All other chemicals used were of the highest grade commercially available.

Preparation of microsomes and cytosol

Male Sprague–Dawley rats (6–7 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). Rats were decapitated and exsanguinated, and the liver, kidneys and lungs immediately removed. Microsomes and cytosol were prepared as described previously (Kamataki and Kitagawa 1973). Briefly, tissues were homogenized in 1.15% (w/v) KCl solution using a Teflon homogenizer and centrifuged at 9000 *g* for 20 min. The supernatant fraction was then centrifuged at 105000 *g* for 1 h to yield both the microsomal pellet and cytosol. The microsomal pellet was

washed once and re-suspended in ice-cold 1.15% (w/v) KCl solution. Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The tissue homogenates were frozen in liquid nitrogen and stored at −80 °C.

FMOs are unstable to heat treatment. Thus, to assess the possibility of FMO being involved in the oxidation reactions, liver microsomes were heat-treated at 45 °C for 5 min as described by Kashiyama *et al.* (1994c).

Analytical procedures

Reduction of flosequinan. The reductase activities for R-FSO, S-FSO and FSO_2 were determined by hplc as the production of FS, R-FSO and S-FSO respectively. A typical reaction mixture (500 μl) consisted of 1 mM substrate, 0.1 M Na,K-phosphate (pH 7.4), 0.1 mM EDTA and a cytosolic fraction (3–5 mg protein/ml). The mixture was pre-incubated at 37 °C for 10 min, and the reaction was then initiated by the addition of an NADPH-generating system (5.0 mM glucose 6-phosphate, 0.5 mM $NADP^+$, 1 unit/ml glucose 6-phosphate dehydrogenase, 5 mM $MgCl_2$), 2-OH-PM (2 mM) or DTT (10 mM) as an electron donor. When necessary, the atmosphere in the reaction tube was replaced with nitrogen, and glucose oxidase (10 units/ml) and 50 mM glucose were also added to the reaction mixture to remove oxygen. The reaction mixture was incubated at 37 °C for 30 min, and then chloroform (3 ml) was added to terminate the reaction, followed by the addition of 0.5 μg (±)-7-chloro-1-methyl-3-methylsulphinyl-4-quinolone as an internal standard. Metabolites were extracted with chloroform. The organic layer (2 ml) was transferred to another tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was dissolved in the solvent used as the mobile phase for hplc as described below and then injected into the hplc system.

S-oxidation. *S*-oxidase activities for FS, R-FSO and S-FSO were determined by hplc as the production of R-FSO, S-FSO and FSO_2 respectively. A typical reaction mixture (200 μl) consisted of 0.1 M phosphate buffer (pH 7.4), 0.1 mM EDTA, 1 mM substrate and the microsomal fraction (0.5–1 mg protein/ml). The mixture was pre-incubated at 37 °C for 3 min, and reactions were then initiated by the addition of the NADPH-generating system. When appropriate, SKF-525A, *n*-octylamine or naphthylthiourea was added at a respective final concentration of 1, 3 or 1 mM. The reaction mixture was incubated at 37 °C for 10 min. All other experimental conditions were the same as for the reductase assay.

Determination of metabolites

The determination of metabolites was performed using the hplc method previously reported by our laboratory (Kashiyama *et al.* 1994a). The hplc system consisted of an HLC-803D system (Tosoh, Tokyo, Japan) and a C-R6A integrator (Shimadzu, Kyoto, Japan). The determination of R-FSO and S-FSO was performed using a chiral-phase column (Chiralcel OD, 4.6 mm i.d. × 250 mm; Daicel Chemical Industries, Tokyo, Japan). Metabolites were eluted with an ethanol–methanol (50:50 v/v) mixture at 0.7 ml/min. A UV-visible absorbance detector (UV-8000; Tosoh) monitored the eluent at 320 nm. Determination of FS and FSO_2 was performed using a reversed-phase column (Inertsil ODS-2, 4.6 mm i.d. × 250 mm; GL Sciences, Tokyo, Japan) equilibrated and delivered with 25%

(v/v) acetonitrile at 1 ml/min. Elution of FSO_2 was detected by the absorbance at 320 nm. FS was detected using a fluorescence detector (RF-530; Shimadzu) with an excitation wavelength of 370 nm and an emission wavelength of 430 nm. The calibration curve was generated for 10–1000 ng per reaction mixture by processing the authentic standard substrates through the entire procedure. The linearity of formation of *S*-oxidized metabolites was confirmed for up to 30 min in the presence of 0.05 and 1 mM substrate and 1 mg protein/ml incubation mixture (Kashiyama *et al*. 1997); while the reduction of R-FSO and S-FSO was linear for up to 60 min in the presence of 1 mM substrate and 5 mg of cytosolic protein/ml.

Kinetic analysis

Enzyme kinetic parameters were estimated by the non-linear least-squares regression analysis of Eadie–Hofstee plots. Substrate concentration varied from 0.02 to 1 mM.

Inhibition of flosequinan metabolism by antibodies to rat NADPH-cytochrome P450 reductase

Antiserum to rat NADPH cytochrome P450 reductase was raised in rabbit as described by Kamataki *et al*. (1976). Immuno-inhibition was measured in the presence of 0, 2.5, 6, 10, 25 or 60 mg antiserum protein per mg of liver microsome protein. The mixture of antiserum and microsomes was incubated for 60 min at 4 °C before incubation. All subsequent reaction conditions as described above.

Results

Enzyme(s) responsible for the reduction of flosequinan

To confirm the proposed metabolic pathways of flosequinan, the metabolism of flosequinan was investigated *in vitro*. Reductase activities in rat cytosol from the liver and from the kidney were examined (table 1). NADPH is known to be an electron donor for reductases. Aldehyde oxidase reduces the sulphoxide group in the presence of 2-OH-PM under an anaerobic condition (Yoshihara and Tatsumi 1985). At a high concentration, DTT can be an electron donor for thioredoxin-dependent sulphoxide reductase (Fukazawa *et al*. 1987). In the present study, under an anaerobic condition using 2-OH-PM as an electron donor, liver cytosol supported higher activity for reduction of R-FSO to FS than for reduction from S-FSO to FS. 2-OH-PM-dependent reduction was also observed in kidney cytosol, but the activity was lower than that in liver cytosol. When DTT was used as an electron donor, the reduction in kidney cytosol was higher than that in liver cytosol (table 1). In kidney cytosol, the reduction of S-FSO to FS was twice as high as that of R-FSO to FS. No activity for reduction of FSO_2 to R-FSO or S-FSO was not detected (data not shown).

Enzyme(s) responsible for S*-oxidation of FS and flosequinan*

As described above, it was demonstrated that FS was produced *in vitro* by cytosolic enzymes. Since, as reported by Kashiyama *et al*. (1994b), FS is not excreted in urine, FS must be the substance oxidized to produce the major final metabolite FSO_2 (figure 1). Thus, to clarify the rate of each *S*-oxidation pathway in rat organs, the NADPH-dependent *S*-oxidase activities in microsomes from the liver, kidney and lung were measured (table 2). Liver microsomes catalysed all four

Table 1. Electron donors for the stereoselective reduction of flosequinan in rat cytosol from both the liver and kidney.

Electron donor	Atmosphere	Reduction (nmol/h/mg protein) R-FSO→ FS	S-FSO→ FS
Liver			
NADPH	air	0.08±0.01	0.11±0.02
NADPH	nitrogen[a]	0.11±0.02	0.08±0.01
2-OH-PM	air	0.04±0.02	0.02±0.00
2-ON-PM	nitrogen[a]	4.6 ±1.9	0.27±0.07
DTT	air	0.15±0.03	0.18±0.03
Kidney			
NADPH	air	0.13±0.01	0.02±0.00
NADPH	nitrogen[a]	0.13±0.01	0.01±0.00
2-OH-PM	air	n.d.	n.d.
2-OH-PM	nitrogen[a]	0.74±0.42	0.09±0.04
DTT	air	0.49±0.08	1.0 ±0.1

[a] Reaction mixture contained 10 units glucose oxidase and 50 mM glucose.

Each value represents the mean±SD from four individual determinations.

NADPH was generated in the mixture of an NADPH-generating system as described in the Material and methods.

2-OH-PM, 2-hydroxypyrimidine; DTT, dithiothreitol; n.d., not detectable (<0.01 nmol/h/mg protein).

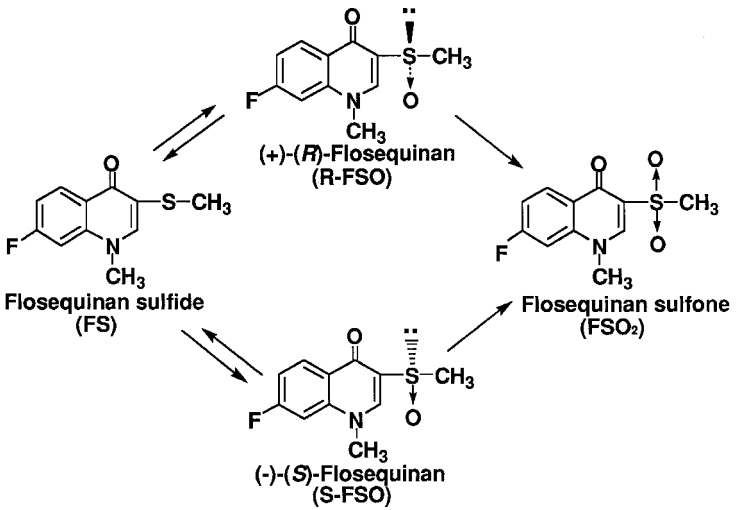

Figure 1. Proposed metabolic pathways for flosequinan in rat.

S-oxidation reactions. The rates of formation of R-FSO and S-FSO from FS were 7–10 times higher than those from R-FSO and S-FSO to FSO_2. On the other hand, microsomes from both the kidney and lung catalysed only the *S*-oxidation of FS to R-FSO, and this stereoselective oxidation activity of microsomes from both the kidney and lung was respectively 26 and 15% of the activity of liver microsomes.

To elucidate the catalytic properties of the *S*-oxidase, the kinetics of these *S*-oxidation reactions were investigated. Typical Eadie–Hofstee plots of the four different *S*-oxidation reactions in liver microsomes are shown in figure 2. The plots of the *S*-oxidation reactions of FS to R-FSO and to S-FSO in liver microsomes each showed a biphasic kinetic behaviour. The *S*-oxidation of R-FSO and S-FSO to

Table 2. Stereoselective *S*-oxidation of flosequinan by microsomes from rat liver, kidney and lung.

Reaction	S-oxidation (nmol/min/mg protein)		
	Liver	Kidney	Lung
FS → R-FSO	3.44±0.25	0.88±0.15	0.50±0.07
FS → S-FSO	2.89±0.65	n.d.	n.d.
R-FSO → FSO_2	0.35±0.05	n.d.	n.d.
S-FSO → FSO_2	0.45±0.06	n.d.	n.d.

Each value represents the mean±SD from six individual determinations.
n.d., Not detectable (<0.01 nmol/min/mg protein).

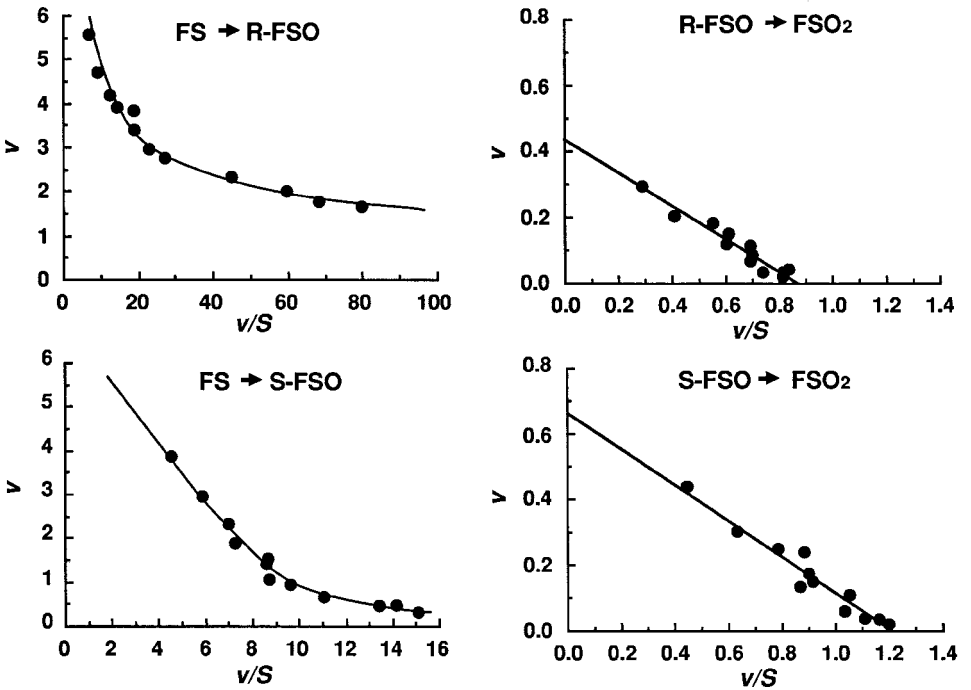

Figure 2. Eadie–Hofstee plots for the *S*-oxidation of flosequinan in rat liver microsomes. Assays were performed as described in the Material and methods. Protein concentration was 0.5 mg/ml for assay of the *S*-oxidation of FS and 1 mg/ml for the assay of the *S*-oxidation of R-FSO and S-FSO. Substrate concentration of FS, R-FSO and S-FSO ranged from 0.02 to 1 mM. *v*, Velocity of product formation (nmol/min/mg protein).

FSO_2 in liver microsomes exhibited a monophasic kinetic behaviour. The kinetic parameters using the two-enzyme kinetic approach (K_{m1}, K_{m2}, $V_{max\,1}$, $V_{max\,2}$), together with the respective ratios or intrinsic clearance ($V_{max\,1}/K_{m1}$, $V_{max\,2}/K_{m2}$) for *S*-oxidation of FS to R-FSO and to S-FSO, and the one-enzyme kinetic approach for *S*-oxidation of R-FSO and S-FSO to FSO_2 (K_m, V_{max}, V_{max}/K_m) are shown in table 3. $V_{max\,1}$ was three times lower than $V_{max\,2}$, whereas K_{m1} was 60 times lower than K_{m2} for the *S*-oxidation of FS to R-FSO. Thus, $V_{max\,1}/K_{m1}$ was calculated as 20 times higher than $V_{max\,2}/K_{m2}$, indicating that an enzyme with a high-affinity component for *S*-oxidation mainly catalysed *S*-oxidation. Comparing the intrinsic clearance (V_{max}/K_{m1}) for FS to R-FSO and to S-FSO, the former was nine times higher than the latter, indicating that the *S*-oxidation of FS to R-FSO was a major pathway of FS

Table 3. Kinetic parameters for the S-oxidation of flosequinan in microsomes from rat liver, kidney and lung.

	Reaction	K_{m1} or K_m (mM)	$V_{max\,1}$ or V_{max} (nmol/min/mg protein)	$V_{max\,1}/K_{m1}$ or V_{max}/K_m (ml/min/mg protein)	$K_{max\,2}$ (mM)	$V_{max\,2}$ (nmol/min/mg protein)	$V_{max\,2}/K_{m2}$ (ml/min/mg protein)
Control							
Liver	FS → R-FSO	0.021	2.5	0.12	1.2	7.4	0.0062
	FS → S-FSO	0.12	1.6	0.013	1.2	5.9	0.0049
	R-FSO → FSO_2	0.40	0.40	0.0010			
	S-FSO → FSO_2	0.44	0.63	0.0014			
Kidney	FS → R-FSO	0.015	0.94	0.063			
Lung	FS → R-FSO	0.021	0.42	0.020			
Heat treatment							
Liver	FS → R-FSO				0.97	6.3	0.0065
	FS → S-FSO	0.14	1.6	0.011	0.80	4.9	0.0061
	R-FSO → FSO_2	0.44	0.29	0.00066			
	S-FSO → FSO_2	0.42	0.48	0.0011			

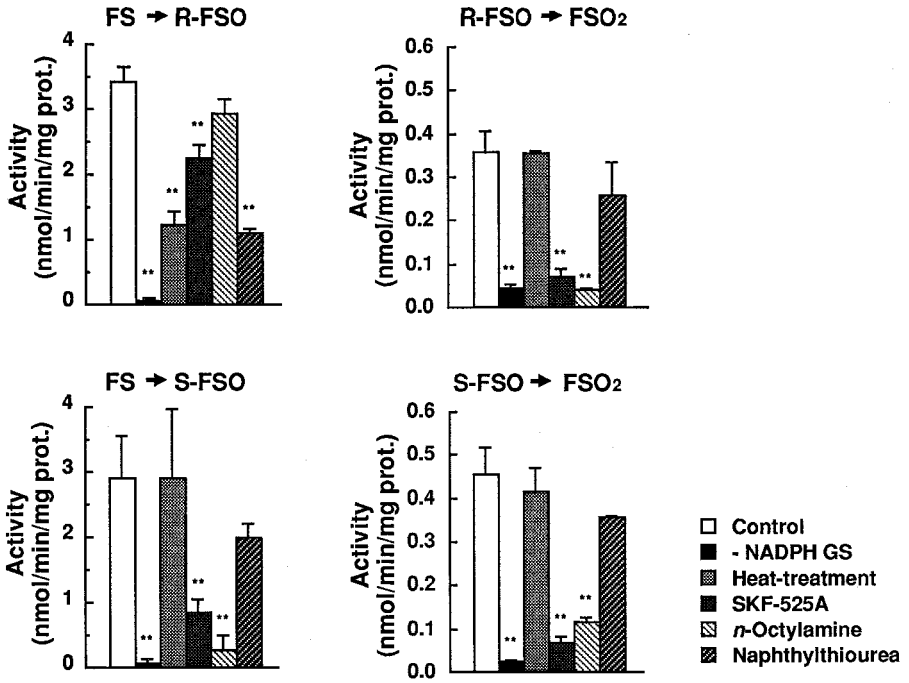

Figure 3. Effects of various modifiers of *S*-oxidase activity in rat liver microsomes. '-NADPH GS' is defined as the absence of an NADPH-generating system in the reaction mixture. Heat treatment of the microsomes was performed at 45 °C for 5 min. The concentrations of SKF-525A, *n*-octylamine and naphthylthiourea added were respectively 1, 3 and 1 mM. Experimental details are described in the text. Values are plotted as the mean ± SD for three-to-six determinations. Results significantly different from the control: $^{**}p < 0.01$.

S-oxidation. The kinetic parameters for the *S*-oxidation of R-FSO or S-FSO to FSO_2 were similar. The intrinsic clearance for the *S*-oxidation of R-FSO and S-FSO to FSO_2 were respectively ~ 100 and ~ 10 times lower than those for the *S*-oxidation of FS to R-FSO and of FS to S-FSO.

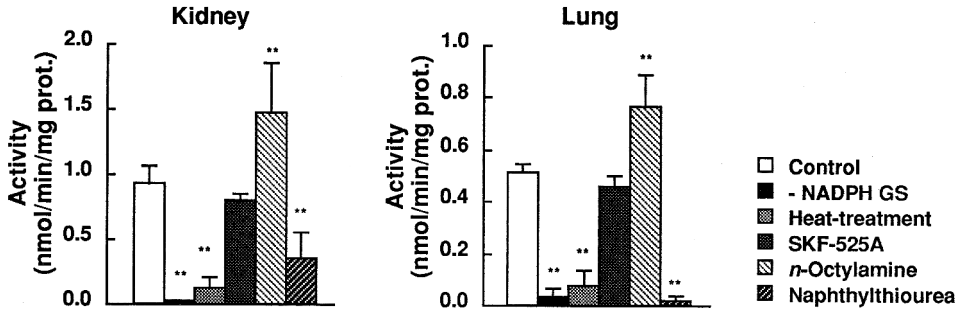

Figure 4. Effects of various modifiers of the *S*-oxidation of FS to R-FSO in rat microsomes from both the kidney and lung. Experimental details are the same as for figure 3. Values are plotted as the mean±SD for three-to-six determinations. Results significantly different from the control: $^{**}p < 0.01$.

On the other hand, the *S*-oxidation of FS to R-FSO in microsomes from both the kidney and lung also showed monophasic kinetics, and the kinetic parameters were calculated using the one-enzyme kinetic approach (table 3). K_m for the *S*-oxidation of FS to R-FSO in microsomes from both the kidney and lung were similar to the K_{m1} seen with liver microsomes.

To clarify further which enzymes are involved in the metabolic pathways, the effects of inhibitors, activators and heat treatment of the microsomes on the *S*-oxidation pathways in liver microsomes were investigated (figure 3). All *S*-oxidation reactions required an NADPH-generating system. The enzyme catalysing the *S*-oxidation of FS to R-FSO was inhibited 30 and 70% by SKF-525A and naphthylthiourea respectively. All *S*-oxidase activities were inhibited by both SKF-525A and *n*-octylamine (>70%), but except for the *S*-oxidation of FS to R-FSO, none of the activities was affected by naphthylthiourea. In addition, heat treatment of the microsomes resulted in a significant decrease (65%) of *S*-oxidation of FS to R-FSO, whereas the same heat treatment did not affect any other *S*-oxidase activities.

To analyse further the effect of heat treatment of the liver microsomes on *S*-oxidase activities, the kinetic parameters of the *S*-oxidation in the heat-treated microsomes were calculated (table 3). The *S*-oxidation of FS to R-FSO in the heat-treated microsomes showed a monophasic kinetic pattern. The high-affinity component seen with untreated microsomes disappeared on heat treatment. The kinetic parameters of other *S*-oxidation pathways were unaffected.

The FS *S*-oxidation to R-FSO in microsomes from both the kidney and lung were decreased by >80% with heat treatment (figure 4). The FS *S*-oxidation to R-FSO was significantly inhibited by naphthylthiourea but not by SKF-525A. *n*-Octylamine enhanced the *S*-oxidase activities in microsomes from both the kidney and lung.

The effects of antibodies to rat NADPH-cytochrome P450 reductase on the *S*-oxidation of flosequinan in microsomes from the liver, kidney and lung were also investigated (figure 5). *S*-oxidase activities in liver microsomes were inhibited ~ 70%, except for the *S*-oxidation of FS to R-FSO (30%). The antibody did not inhibit the *S*-oxidation of FS to R-FSO in microsomes from both the kidney and lung.

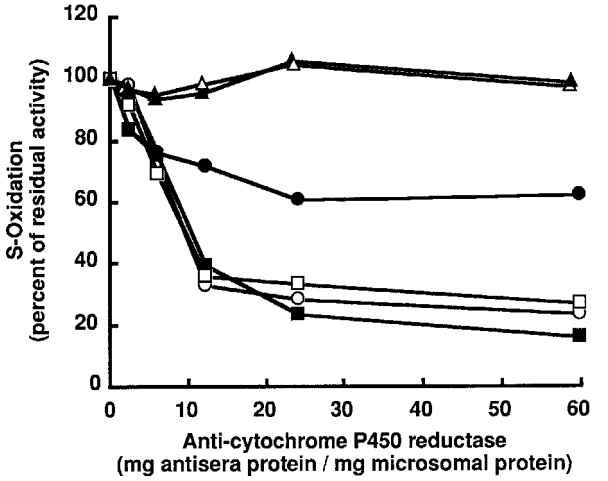

Figure 5. Immuno-inhibition of *S*-oxidation by antiserum to NADPH-cytochrome P450 reductase. Formation of R-FSO from FS (●), S-FSO from FS (○), FSO_2 from R-FSO (■) and FSO_2 from S-FSO (□) by liver microsomes and formation of R-FSO from FS by kidney (△) and lung (▲) microsomes from rat are shown. Results are the amount of activity remaining relative to the untreated control. Activities in the absence of antibody are the same as those shown in table 2. Data are the mean of duplicate determinations.

Discussion

The reduction of drugs has not been studied as much as their *S*-oxidation. It has been suggested that enzymes such as aldehyde oxidase (Tatsumi *et al.* 1983) and thioredoxin-linked enzyme systems (Aymard *et al.* 1979, Fukazawa *et al.* 1987, Anders *et al.* 1981) are involved in the reduction of sulphoxides to sulphides. Although these enzymes have been purified from liver and kidney cytosol, no information is yet available on stereoselectivity in the metabolism of foreign compounds. To our knowledge, the present paper is the first to describe the stereoselective reduction of sulphoxides.

Aldehyde oxidase usually reduces heterocyclic xenobiotics, such as 2-OH-PM and *N*-methylnicotinamide, in the presence of an electron donor under anaerobic conditions (Yoshihara and Tatsumi 1985). In the present study, it is possible that aldehyde oxidase is involved in the reduction of R-FSO and S-FSO to FS, since those activities were detectable when cytosol from both the liver and kidney was used in the presence of 2-OH-PM. The reduction of R-FSO to FS was 17 times higher than that of S-FSO to FS in liver microsomes, suggesting that aldehyde oxidase predominantly reduced R-FSO to FS.

In the case of the thioredoxin-linked enzyme system, NADPH serves to maintain thioredoxin in its reduced form, and thioredoxin, in turn, acts as a hydrogen donor to a sulphoxide reductase (Anders *et al.* 1981). This thioredoxin system can be efficiently replaced by a high concentration of DTT (Anders *et al.* 1980). In the present study, it is possible that the thioredoxin-linked enzyme system is also involved in the reduction of R-FSO and S-FSO to FS in the kidney.

The *S*-reduction of R-FSO and S-FSO to FS was confirmed *in vitro*, even although the level of reductase activity was relatively low in comparison with *S*-oxidase activity. For example, sulindac reductase activity of aldehyde oxidase in the guinea pig liver and that of the thioredoxin-linked enzyme system in rat kidney

cytosol (Anders *et al.* 1981, Tatsumi *et al.* 1983) were respectively >100 and 20 times higher than the flosequinan reductase activity seen in this study. It has been reported that sulindac is mainly reduced in the organs, whereas sulphinpyrazone is mainly reduced by gut flora (Renwick *et al.* 1986). Flosequinan would likely be mainly reduced by gut flora rather than enzymes in the organs since the extent of chiral interconversion when flosequinan was given to rat orally was greater than when it was given intravenously (Kashiyama *et al.* 1994b, d). No reduction of FSO_2 to R-FSO or S-FSO was observed in the present study, supporting the finding that FSO_2 was not reduced when it was given to rat intravenously (Kashiyama *et al.* 1994b).

Liver microsomes catalysed four *S*-oxidation pathways in flosequinan metabolism. The results of the present study suggest that the *S*-oxidation of FS to R-FSO is catalysed by both P450 and FMO, and that the other three *S*-oxidation reactions are catalysed by P450 but not by FMO. Interestingly, microsomes from both the kidney and lung exclusively catalysed the *S*-oxidation of FS to R-FSO. The involvement of FMO was strongly implicated in the present study.

Eadie–Hofstee plot analysis indicated that the *S*-oxidation of FS to R-FSO in liver microsomes was catalysed by at least two distinct enzymes. One is perhaps an FMO and the other a P450. It is suggested that an FMO was responsible for the high-affinity enzyme in *S*-oxidation since heat treatment caused the disappearance of one of the two enzymes with a low K_m in Eadie–Hofstee plots. $V_{max\,1}/K_{m1}$ for FMO was 20 times higher than $V_{max\,2}/K_{m2}$. Therefore, FMO are considered mainly to catalyse the *S*-oxidation of FS to R-FSO in the liver *in vivo*. This *S*-oxidation in kidney and lung microsomes is also suggested to be catalysed by FMO.

The present authors previously reported that rat hepatic FMO1A1 expressed in yeast only catalysed the *S*-oxidation of FS to R-FSO (Kashiyama *et al.* 1994c). K_m for the reaction of FS to R-FSO seen with microsomes from the liver, kidney and lung were all similar to that seen using rat FMO1A1 expressed in yeast (33 μM). The presence of multiple FMO isoforms with different catalytic properties has been reported (Lawton and Philpot 1993, Ziegler 1993), and immunoblot and Northern blot analyzes have suggested that one or more similarly structured FMO(s) are expressed in microsomes from rat liver, kidney and lung (Itoh *et al.* 1993, Sausen *et al.* 1993). Considering these findings, one or more FMO(s) with the same or similar catalytic properties are likely expressed in microsomes in rat liver, kidney and lung, and they might catalyse the stereoselective *S*-oxidation of RS to R-FSO in these organs. The *S*-oxidation pathway of FS to S-FSO in liver microsomes was also shown to be catalysed by at least two distinct enzymes, indicating that isozymes of P450 were responsible for the reaction of FS to S-FSO.

It has previously been reported that the plasma concentration of R-FSO was higher than that of S-FSO when FS was given to rat either orally and intravenously (Kashiyama *et al.* 1994b). $V_{max\,1}/K_{m1}$ for the *S*-oxidation of FS to R-FSO was higher than that for *S*-oxidation of FS to S-FSO, indicating that FS was preferentially oxidized to R-FSO. V_{max}/K_m for *S*-oxidation of R-FSO and S-FSO to FSO_2 were similar but lower than the $V_{max\,1}/K_{m1}$ for the *S*-oxidation of FS to R-FSO and S-FSO. These findings suggest that the metabolism of R-FSO and S-FSO to FSO_2 is the rate-limiting step. The results of the present *in vitro* study are consistent with the pharmacokinetic behaviour of flosequinan *in vivo*. Thus, the kinetic analysis of the metabolism of drugs *in vitro* is expected to be useful in understanding *in vivo* metabolism.

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