Involvement of CYP2C9 and UGT2B7 in the metabolism of zaltoprofen, a nonsteroidal anti-inflammatory drug, and its lack of clinically significant CYP inhibition potential

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Aims To identify the cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) isoforms responsible for the formation of the primary metabolite(s) of zaltoprofen, and to predict possible drug interactions by investigating the inhibition of CYP isoforms *in vitro*.

Methods The metabolism of zaltoprofen was studied *in vitro* using recombinant CYP and UGT isoform cDNA-expression systems. The effects of selective isoform inhibitors on zaltoprofen metabolism were studied using human liver microsomes. The inhibitory effects of zaltoprofen on the metabolism of selective probe substrates for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were also determined in human liver microsomes.

Results Zaltoprofen was extensively metabolized by CYP2C9 and UGT2B7. CYP2C9 catalysed sulphoxidation but not hydroxylation of zaltoprofen. In the human liver microsomal metabolism study, zaltoprofen metabolism was markedly inhibited by sulphaphenazole, a selective inhibitor of CYP2C9. In the drug interaction study, negligible inhibition (< 15%) of the activities of CYP1A2, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 was apparent at 5 µg ml⁻¹, the maximum plasma concentration observed in humans after oral administration of an 80 mg zaltoprofen tablet. However, zaltoprofen inhibited CYP2C9 by 26% at 5 µg ml⁻¹. At higher concentrations, zaltoprofen produced some inhibition of CYP2C9 (IC₅₀ = 19.2 µg ml⁻¹; 64.4 µM) and CYP3A4 (IC₅₀ = 53.9 µg ml⁻¹; 181 µM). The free drug concentrations in plasma (0.02 µg ml⁻¹, 67.0 nM) at the C_{max} of the clinically effective doses are much lower than the IC₅₀ values corrected for the nonspecific binding ratio of zaltoprofen to microsomal protein (15.5 µg ml⁻¹ for CYP3A4, 49.5 µg ml⁻¹ for CYP2C9). Furthermore, the maximum free drug concentrations in the hepatic intracellular was calculated to be 0.068 µg ml⁻¹ and the increase in the AUC in the presence of zaltoprofen was estimated to be only 0.4% for CYP2C9 substrates and 0.1% for CYP3A4 substrates, respectively.

Conclusions Zaltoprofen is predominantly metabolized by CYP2C9 and UGT2B7, and is considered unlikely to cause significant drug interactions *in vivo* when coadministered with CYP substrates at clinically effective doses.

Keywords: CYP2C9, human liver microsomes, *in vitro* metabolism, UGT2B7, zaltoprofen

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Introduction

Zaltoprofen, ($\pm$)-2-(10,11-dihydro-10-oxodibenzo [*b, f*] thiepin-2-yl)propionic acid (Figure 1), belongs to the therapeutic class of nonsteroidal anti-inflammatory drugs (NSAIDs), which are used in the treatment of rheumatoid arthritis and osteoarthritis as well as to relieve inflammation and pain after surgery, injury and tooth extraction [1].

After oral administration of zaltoprofen to humans, 62% of the dose is excreted as conjugates in the urine and only 3% is excreted as unchanged compound by this route [2]. Zaltoprofen is also biotransformed to S-oxide-zaltoprofen (M-2), 10-hydroxy-zaltoprofen (M-3) and S-oxide-10-hydroxy-zaltoprofen (M-5) in humans, and conjugates of M-2 and M-3 are excreted in the urine, although urinary levels of each of these metabolites account for less than 10% of the dose [2].

The existence and functions of cytochrome P450 (CYP) isoforms in human liver microsomes are well known [3–7]. Numerous CYP isoforms have now been cloned and expressed in various cells using cDNA technology, and these systems have become available for the evaluation of drug metabolism [8, 9]. Furthermore, many reports have described serious adverse effects caused by drug–drug interactions resulting from the inhibition of CYP isoforms [5, 6, 9]. The selective substrates of many individual CYP isoforms have now been identified, and potential drug–drug interactions can be predicted by studying the inhibition of their metabolism *in vitro* [6, 9–12]. Conjugation with glucuronide is another major metabolic pathway for drugs, and is catalysed by UDP-glucuronosyltransferase (UGT) phase II enzymes [13, 14]. Recombinant human UGTs have also been used to study metabolism *in vitro* [13, 14]. However, the CYP and UGT enzymes involved in the metabolism of zaltoprofen have not previously been identified.

In the present study, the principal CYP and UGT isoforms involved in the metabolism of zaltoprofen were investigated using human liver microsomes and cDNA-expression systems. Potential interactions between zaltoprofen and other drugs that act as selective substrates for CYPs were also investigated by determining any inhibition of their metabolism in human liver microsomes.

Methods

Chemicals

Zaltoprofen (Lot Nos. P390 and 3007), [14 C]-zaltoprofen, S-oxide-zaltoprofen (M-2; 2-(10,11-dihydro-5,10-dioxodibenzo [*b, f*] thiepin-2-yl) propionic acid), 10-hydroxy-zaltoprofen (M-3; sodium 2-(10,11-dihydro-

Zaltoprofen

M-2 (S-oxide-zaltoprofen)

M-3 (10-hydroxy-zaltoprofen)

M-5 (S-oxide-10-hydroxy-zaltoprofen)

Figure 1 Chemical structures of zaltoprofen and its metabolites.

10-hydroxydibenzo [*b, f*] thiepin-2-yl) propionate) and S-oxide-10-hydroxy-zaltoprofen (M-5; 2-(10,11-dihydro-10-hydroxy-5-oxodibenzo [*b, f*] thiepin-2-yl) propionic acid) were supplied from Nippon Chemiphar Co., Ltd. (Tokyo, Japan); (Figure 1).

α -Naphthoflavone, sulphaphenazole, tranlylcypromine, quinidine and troleandomycin were purchased from Sigma (MO, USA), furafylline was purchased from Ultrafine Chemicals Ltd (Salford, UK), and ketoconazole was purchased from ICN Biomedicals Inc (CA, USA). Mefenamic acid and omeprazole were purchased from Wako Pure Chemical Industry (Osaka, Japan), diethyldithiocarbamate was purchased from Sigma and Wako.

cDNA-expressed microsomes from baculovirus-infected insect cells for human CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP2C9*1(Arg144), CYP2C19, CYP2D6*1(Val374), CYP2E1, CYP3A4, flavin-containing monooxygenase (FMO) 3, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15, and CYP and UGT insect cell control SUPERSOMES® were obtained from Gentest (MA, USA).

Pooled human liver microsomes were obtained from Gentest (Lot No. 16) and Tissue Transformation Technologies (Lot No. HHM-0281, NJ, USA) with the approval of the local ethics committee.

All other reagents used were of at least analytical grade.

Metabolism of zaltoprofen in CYP and UGT cDNA-expression systems

For the metabolic study using CYP isoforms, the reaction mixture (total volume, 0.5 ml) consisted of 40 mM Tris-HCl buffer (pH 7.4) containing 10 mM β -NADPH, 1 mM EDTA and 6 mM $MgCl_2$, 5 $\mu g\ ml^{-1}$ zaltoprofen, and 2 mg ml^{-1} microsomal protein from the enzyme cDNA-expression system (160 pmol CYP ml^{-1}). The mixture was incubated at 37 °C for 2 h. For the metabolic study using UGT isoforms, the reaction mixture (total volume of 0.25 ml) consisted of 125 mM Tris-HCl buffer (pH 7.4) containing 0.004% Triton X, 4 mM $MgCl_2$, 0.8 mM UDP-glucuronic acid (UDPGA), 1 $\mu g\ ml^{-1}$ zaltoprofen, and 1 mg ml^{-1} microsomal protein from the enzyme cDNA-expression system. The mixtures were activated by preincubation at 37 °C for 20 min without zaltoprofen. The reaction was then initiated by adding zaltoprofen, and incubation was continued for 4 h.

The reaction was terminated by adding acetonitrile containing mefenamic acid as an internal standard, and the mixture was centrifuged at 9000g for 10 min. The supernatant was injected onto a high-performance liquid chromatography (h.p.l.c.) column, and the disappearance of zaltoprofen was determined.

Reaction mixtures containing UGT2B7 were also hydrolysed by overnight incubation at 37 °C with β -glucuronidase (type H1, 1000 unit ml^{-1}) in 0.1 M acetate buffer (pH 5.0). After incubation, the samples were treated as described above. The experiments for each isoform were performed in triplicate.

To identify the metabolites of zaltoprofen, the above procedure was carried out using a zaltoprofen concentration of 20 $\mu g\ ml^{-1}$ and 500 pmol ml^{-1} CYP2C9 at 37 °C. The reaction was allowed to proceed for 20 min, and was terminated by adding 1 M acetate buffer (pH 4.5) and dichloromethane. After adding methyl *p*-hydroxybenzoate as an internal standard, the mixture was centrifuged at 2000g for 10 min. The organic phase was evaporated to dryness under a stream of nitrogen gas, and the residue was dissolved in the mobile phase used for h.p.l.c.

Effects of chemical inhibitors on the metabolism of zaltoprofen by CYP in human liver microsomes

Chemical enzyme inhibitors were added to the reaction medium in order to determine the quantitative contribution of individual CYP isoforms to zaltoprofen metabolism. The reaction mixture (total volume, 0.5 ml) consisted of 0.1 M Tris-0.1 M HCl buffer (pH 7.4) containing 10 mM β -NADPH, 1 mM EDTA and 6 mM $MgCl_2$. In the experiment to determine the effect of diethyldithiocarbamate, preincubation was carried out at 37 °C for 15 min before initiating the reaction by adding zaltoprofen. The final concentrations of inhibitors were based on the K_i values published in the literature: α -naphthoflavone (0.04, 0.2, 1 μM ; CYP1A1/2) [8], tranlylcypromine (0.08, 0.4, 2 μM ; CYP2A6) [7], sulphaphenazole (0.2, 1, 5 μM ; CYP2C9) [3–5,8], omeprazole (0.4, 2, 10 μM ; CYP2C19) [5, 6], quinidine (0.04, 0.2, 1 μM ; CYP2D6) [3, 5, 8], diethyldithiocarbamate (1, 5, 25 μM ; CYP2E1) [3], ketoconazole (0.04, 0.2, 1 μM ; CYP3A4) [3, 4].

H.p.l.c. analysis of zaltoprofen and its metabolites

The zaltoprofen content of the mixtures was determined by h.p.l.c. system using an L-Column ODS (4.6 mm i.d. $\times$ 250 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan) with a guard column, CAPCELL PAK C18 UG120 (4.0 mm i.d. $\times$ 10 mm; Shiseido, Tokyo, Japan) at 35 °C. The mobile phase was acetonitrile: purified water: acetic acid (60 : 40 : 1, v/v/v) and was pumped at a flow-rate of 0.7 ml min^{-1} . The eluate was monitored by a u.v. detector set at 330 nm. In the experiment to identify zaltoprofen metabolites, zaltoprofen and M-2 were simultaneously determined on an analytical column, CAPCELL PAK C18 UG120, 4.6 mm i.d. $\times$ 250 mm, with a guard column, CAPCELL PAK C18 UG120, 4.0 mm i.d. $\times$ 10 mm at 35 °C. The mobile phase was 65 mM NaH_2PO_4 containing 5 mM tetrabutylammonium chloride (adjusted to pH 6.0 with triethylamine): acetonitrile (35 : 15, v/v) and was pumped at a flow-rate of 1 ml min^{-1} . The eluate was

monitored by a u.v. detector set at 270 nm. The within-run and between-run accuracy and precision of these assays did not deviate by more than 15% from the nominal concentration (20% at the limit of quantification), and the analytes remained stable for at least 48 h in the final sample solution at room temperature.

Inhibition of the metabolism of isoform-selective substrates by zaltoprofen

The following marker substrate activities were used to evaluate the potential inhibitory effects of zaltoprofen on CYP isoforms in human liver microsomes: 7-ethoxyresorufin O-deethylase (CYP1A2) [15], tolbutamide methyl hydroxylase (CYP2C9) [16], S-mephenytoin 4'-hydroxylase (CYP2C19) [17], debrisoquine 4-hydroxylase (CYP2D6) [18], lauric acid 11-hydroxylase (CYP2E1) [19] and testosterone 6 β -hydroxylase (CYP3A4) [20]. The experiments were conducted at zaltoprofen concentrations of 5, 15 and 50 $\mu\text{g ml}^{-1}$ for CYP1A2, CYP2C19, CYP2D6 and CYP2E1, and 5, 10, 20, 35, 50 and 75 $\mu\text{g ml}^{-1}$ for CYP2C9 and CYP3A4. Inhibition by the selective chemical inhibitors furafylline (30 μM ; CYP1A2), sulphaphenazole (20 μM ; CYP2C9), tranilcypromine (100 μM ; CYP2C19), quinidine (5 μM ; CYP2D6), diethyldithiocarbamate (300 μM ; CYP2E1) and troleandomycin (101 μM ; CYP3A4) were also investigated. To determine the IC_{50} values (50% inhibition of the enzymatic activity observed in the absence of the inhibitor) for CYP2C9 and CYP3A4, activity data obtained from the following equation were analysed by nonlinear regression analysis (WinNonlin, Pharsight Corp., USA):

$$v = v_0 - (v_0 \times [\text{I}]) / (\text{IC}_{50} + [\text{I}]),$$

where v_0 is the velocity without the inhibitor, v is the observed velocity and $[\text{I}]$ is the inhibitor concentration.

Prediction of the inflow concentration (I_{in}) of the inhibitor into the liver after oral administration can be expressed as follows [10]:

$$I_{\text{in}} = I_{\text{max}} + (k_a \times \text{Dose} \times F_a) / Q_h$$

where I_{max} is the maximum concentration of the inhibitor in the blood, k_a is the first-order rate constant for gastrointestinal absorption, F_a is the fraction absorbed from the gastrointestinal tract into the portal vein, and Q_h is the hepatic blood flow.

I_{max} is calculated as follows:

$$I_{\text{max}} = C_{\text{max}} \times R_{\text{BS}}$$

where C_{max} is the maximum concentration of the inhibitor in the plasma and R_{BS} is the ratio of the concentration in the blood to that in the serum concentration.

The free concentration of zaltoprofen in the liver ($I_{\text{u,liver}}$) can be calculated as follows:

$$I_{\text{u,liver}} = I_{\text{in}} \times f_{\text{plasma}} \times R_{\text{LB}} / R_{\text{BS}}$$

where f_{plasma} is the unbound fraction in the plasma and R_{LB} is the ratio of the zaltoprofen concentration in the liver to that in the blood.

The data on the zaltoprofen used in the above calculations were obtained from the following sources. C_{max} ($= 5 \mu\text{g ml}^{-1}$) and k_a ($= 1.84 \text{ h}^{-1}$) values were determined after an oral dose of 80 mg zaltoprofen [2], f_{plasma} ($= 0.004$) was obtained from a report by Sano *et al.* [21], R_{BS} ($= 0.5$) and R_{LB} ($= 2.1$) from rat radioisotope tracer experiments [22], and Q_h ($= 1610 \text{ ml min}^{-1}$) from Ito *et al.* [10]. The F_a value for zaltoprofen was taken to be 1.

Determination of nonspecific binding of zaltoprofen to liver microsomes

The nonspecific binding of zaltoprofen to the human liver microsomal fraction was determined by an ultracentrifugation method. [^{14}C]-Zaltoprofen (at concentrations of 5, 20 and 75 $\mu\text{g ml}^{-1}$) was mixed with human liver microsomes in 50 mM Tris-HCl buffer (pH 7.4). The concentrations of microsomal protein were 0.5 and 2 mg ml^{-1} , which were those used for the determination of IC_{50} values of CYP3A4 and CYP2C9, respectively.

The mixtures were incubated at 37 °C for 5 min, then centrifuged at 105 000g at 10 °C for 1 h. The radioactivity of zaltoprofen was determined by liquid scintillation counting (2500TR, Packard, Meriden, CT, USA). Binding to the microsomal protein was calculated using the equation:

$$B = (1 - \text{Sup}/\text{Mix}) \times 100$$

where B is the binding (%), Sup is the d min^{-1} in the supernatant after centrifugation, and Mix is the d min^{-1} in the mixture before centrifugation.

Furthermore, the IC_{50} value corrected by unbound microsomal fraction ($\text{IC}_{\text{u},50}$) was calculated using the equation:

$$\text{IC}_{\text{u},50} = \text{IC}_{50} \times (100 - B) / 100$$

Results

Incubation with human CYP isoforms in a cDNA-expression system

During incubation for 2 h with microsomes from baculovirus-infected insect cells containing human CYPs, the most obvious disappearance of zaltoprofen, a 53.6% reduction compared with initial values, was observed with microsomes expressing CYP2C9 (Figure 2). The percentage decreases produced by CYP3A4 and CYP1A1 were 13.1% and 11.8%, respectively. The degree of metabolism produced by other CYP isoforms

Figure 2 Metabolism of zaltoprofen by CYP and FMO3 isoforms in a cDNA-expression system. R; CYP reductase; b5; Cytochrome b5. The values show the percentage reduction from initial values (mean $\pm$ s.d., $n = 3$). The microsomal protein concentration was 2 mg ml^{-1} ($160 \text{ pmol CYP ml}^{-1}$).

was equivalent to that observed with control microsomes ($< 10\%$ reduction from initial zaltoprofen). Metabolism by FMO3 enzyme was also negligible.

In the experiment to characterize zaltoprofen metabolism by CYP2C9, M-2 was identified as the predominant metabolite, and neither M-3 nor M-5 was observed on the chromatogram (Figure 3). The velocity of M-2 formation was $2.34 \pm 0.08 \text{ pmol pmol}^{-1}$ of CYP min^{-1} (mean $\pm$ s.d., $n = 3$), which was equivalent to the disappearance rate of zaltoprofen ($2.23 \pm 0.13 \text{ pmol pmol}^{-1}$ of CYP min^{-1}).

Incubation with human UGT isoforms in a cDNA-expression system

During incubation for 4 h with microsomes expressing UGT, the most obvious disappearance of zaltoprofen, a 53.0% reduction from the initial values, was observed with microsomes expressing UGT2B7 (Figure 4). The degree of metabolism observed with other UGT isoforms was equivalent to that produced control microsomes ($< 10\%$ reduction from initial zaltoprofen). After enzymatic hydrolysis of the UGT2B7 incubation mixture, the proportion of zaltoprofen metabolized was drastically decreased to 14.3%.

Inhibitory effects of isoform-selective chemical inhibitors on the metabolism of zaltoprofen by human liver microsomes

The effects of selective CYP inhibitors on zaltoprofen metabolism in human liver microsomes are shown in Table 1. After incubation for 30 min in the absence of chemical inhibitors, the rate of disappearance of zalto-

Figure 3 H.p.l.c. profile of zaltoprofen metabolism by human CYP2C9*1 in a cDNA-expression system. Analysis of the sample obtained after incubation for 20 min with CYP2C9*1 ($500 \text{ pmol CYP ml}^{-1}$).

Figure 4 Metabolism of zaltoprofen by human UGT isoforms in a cDNA-expression system. The values show the percentage reduction from initial values (mean $\pm$ s.d., $n = 3$). The microsomal protein concentration was 1 mg ml^{-1} .

profen from the reaction mixture was between 0.279 and $0.292 \text{ nmol min}^{-1} \text{ mg}^{-1}$ of protein, and about 50% of the initial zaltoprofen content was metabolized.

This metabolism of zaltoprofen was inhibited by sulphaphenazole in a concentration-dependent manner, decreasing to 14.6% of the control level with $5 \text{ }\mu\text{M}$ sulphaphenazole. Zaltoprofen metabolism was also slightly decreased with $25 \text{ }\mu\text{M}$ diethyldithiocarbamate (to 83.5% of control), $10 \text{ }\mu\text{M}$ omeprazole (to 86.5%) and $1 \text{ }\mu\text{M}$

Table 1 Effect of selective CYP inhibitors on the metabolism of zaltoprofen in human liver microsomes.

Inhibitor	$\mu\text{mol l}^{-1}$	% of control
a-Naphthoflavone (CYP1A2)	0.04	97.9 $\pm$ 3.9
	0.2	98.3 $\pm$ 2.2
	1	96.1 $\pm$ 4.0
Tranlycypromine (CYP2A6)	0.08	99.9 $\pm$ 1.7
	0.4	98.2 $\pm$ 2.9
Sulfaphenazole (CYP2C9)	2	97.9 $\pm$ 1.3
	0.2	72.7 $\pm$ 1.6
	1	33.1 $\pm$ 1.1
Omeprazole (CYP2C19)	5	14.6 $\pm$ 2.7
	0.4	96.3 $\pm$ 3.5
	2	94.3 $\pm$ 1.5
Quinidine (CYP2D6)	10	86.5 $\pm$ 2.5
	0.04	95.7 $\pm$ 4.4
	0.2	94.5 $\pm$ 0.4
Diethyldithiocarbamate (CYP2E1)	1	95.5 $\pm$ 2.4
	1	94.6 $\pm$ 2.1
	5	94.0 $\pm$ 1.9
Ketoconazole (CYP3A4)	25	83.5 $\pm$ 1.7
	0.04	92.5 $\pm$ 3.9
	0.2	92.7 $\pm$ 0.3
	1	91.1 $\pm$ 1.4

The values are mean $\pm$ s.d. ($n = 3$).

ketoconazole (to 91.1%). The other inhibitors tested did not inhibit zaltoprofen metabolism, which remained at over 95% of the control level even with high concentrations of inhibitors.

Effect of zaltoprofen on CYP isoform activities in human liver microsomes

The potential of zaltoprofen to inhibit the catalytic activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 was assessed by measuring the metabolism of marker substrates (Table 2). CYP1A2, CYP2C19, CYP2D6 and CYP2E1 were inhibited by less than 15% with zaltoprofen 5 $\mu\text{g ml}^{-1}$, and by less than 21.1% with 50 $\mu\text{g ml}^{-1}$. On the other hand, zaltoprofen inhibited the activity of CYP2C9, by 25.6% at 5 $\mu\text{g ml}^{-1}$ and 75.3% at 50 $\mu\text{g ml}^{-1}$. Furthermore, although the activity of CYP3A4 was decreased by only 3.6% with 5 $\mu\text{g ml}^{-1}$ of zaltoprofen, the reduction was 48.8% with 51 $\mu\text{g ml}^{-1}$. IC_{50} values for these two isoforms were determined as 19.2 $\mu\text{g ml}^{-1}$ (64.4 μM) for CYP2C9 and 53.9 $\mu\text{g ml}^{-1}$ (181 μM) for CYP3A4.

Nonspecific binding of zaltoprofen to human liver microsomal protein

The nonspecific binding of zaltoprofen was determined as 4.0–8.2% at 0.5 mg protein ml^{-1} , and 16.7–19.5%

Table 2 Effect of zaltoprofen on CYP activities in human liver microsomes.

		% of inhibition
CYP1A2 (7-Ethoxyresorufin O-deethylase activity)		
Zaltoprofen	5 $\mu\text{g ml}^{-1}$	0.0 $\pm$ 0.0
	15 $\mu\text{g ml}^{-1}$	1.3 $\pm$ 2.3
	50 $\mu\text{g ml}^{-1}$	5.8 $\pm$ 5.2
Furafylline	30 $\mu\text{mol l}^{-1}$	100.0 $\pm$ 0.0
CYP2C9 (Tolbutamide methyl hydroxylase activity)		
Zaltoprofen	5 $\mu\text{g ml}^{-1}$	25.6 $\pm$ 3.6
	20 $\mu\text{g ml}^{-1}$	51.0 $\pm$ 0.5
	50 $\mu\text{g ml}^{-1}$	75.3 $\pm$ 6.3
Sulphaphenazole	20 $\mu\text{mol l}^{-1}$	85.1 $\pm$ 4.8
CYP2C19 (S-Mephenytoin 4'-hydroxylase activity)		
Zaltoprofen	5 $\mu\text{g ml}^{-1}$	14.8 $\pm$ 5.8
	15 $\mu\text{g ml}^{-1}$	8.2 $\pm$ 5.2
	50 $\mu\text{g ml}^{-1}$	16.6 $\pm$ 4.7
Tranlycypromine	100 $\mu\text{mol l}^{-1}$	74.5 $\pm$ 7.9
CYP2D6 (Debrisoquine 4-hydroxylase activity)		
Zaltoprofen	5 $\mu\text{g ml}^{-1}$	1.4 $\pm$ 1.5
	15 $\mu\text{g ml}^{-1}$	2.9 $\pm$ 2.5
	50 $\mu\text{g ml}^{-1}$	4.6 $\pm$ 2.2
Quinidine	5 $\mu\text{mol l}^{-1}$	60.6 $\pm$ 2.6
CYP2E1 (Lauric 11-hydroxylase activity)		
Zaltoprofen	5 $\mu\text{g ml}^{-1}$	6.9 $\pm$ 3.2
	15 $\mu\text{g ml}^{-1}$	12.9 $\pm$ 6.8
	50 $\mu\text{g ml}^{-1}$	21.1 $\pm$ 0.2
Diethyldithiocarbamate	300 $\mu\text{mol l}^{-1}$	56.6 $\pm$ 1.4
CYP3A4 (Testosterone 6 β -hydroxylase activity)		
Zaltoprofen	5 $\mu\text{g ml}^{-1}$	3.6 $\pm$ 4.2
	20 $\mu\text{g ml}^{-1}$	26.7 $\pm$ 1.6
	51 $\mu\text{g ml}^{-1}$	48.8 $\pm$ 2.4
Troleandomycin	101 $\mu\text{mol l}^{-1}$	68.9 $\pm$ 2.9

The values are mean $\pm$ s.d. ($n = 3$).

Table 3 Nonspecific binding of zaltoprofen to human liver microsomal protein (%)

Zaltoprofen ($\mu\text{g ml}^{-1}$)	Microsomal protein (mg ml^{-1})	
	0.5	2
5	8.2 $\pm$ 1.8	19.5 $\pm$ 0.7
20	5.2 $\pm$ 0.9	16.8 $\pm$ 1.3
75	4.0 $\pm$ 1.5	16.7 $\pm$ 1.1

The values are mean $\pm$ s.d. ($n = 3$).

at 2 mg protein ml^{-1} , respectively (Table 3). A slight decrease in binding to microsomal subcellular fractions with increase in total zaltoprofen concentration was observed. The minimum free fraction of zaltoprofen was 0.918 at 0.5 mg protein ml^{-1} and 0.805 at 2 mg protein ml^{-1} , respectively. Therefore, the IC_{50} values corrected for the nonspecific binding ratio were calculated to be 15.5 $\mu\text{g ml}^{-1}$ (52.0 μM) for CYP2C9 and 49.5 $\mu\text{g ml}^{-1}$ (166 μM) for CYP3A4.

Prediction of the drug interaction in clinical setting

Zaltoprofen is 99.6% bound to human plasma proteins; the free fraction in the plasma is therefore only 0.4% [21]. At $C_{\max}$ ($5 \mu\text{g ml}^{-1}$), the concentration of free zaltoprofen in the plasma is only $0.02 \mu\text{g ml}^{-1}$ (67.0 nM). Thus, the IC_{50} values corrected by nonspecific binding ratio for CYP2C9 and CYP3A4 are approximately 800 times and 2500 times higher than the free plasma concentration at $C_{\max}$, respectively. The maximum inflow concentration of zaltoprofen into the liver (I_{in}) and the unbound concentration in the hepatic intracellular ($I_{\text{u,liver}}$) were calculated to be 4.02 and $0.068 \mu\text{g ml}^{-1}$, respectively. Comparing the $I_{\text{u,liver}}$ to the IC_{50} corrected by nonspecific binding ratio, the increase in the AUC in the presence of zaltoprofen was estimated to be only 0.4% for CYP2C9 substrates and 0.1% for CYP3A4 substrates, respectively.

Discussion

To identify the CYP and UGT isoforms involved in the metabolism of zaltoprofen, studies were conducted using human liver microsomes and recombinant human enzymes. Zaltoprofen was extensively metabolized by CYP2C9 expressed in microsomes derived from baculovirus-infected insect cells. In the human liver microsomal metabolism study, zaltoprofen metabolism was markedly inhibited by sulphaphenazole, a selective inhibitor of CYP2C9. CYP2C9 is responsible for the metabolism of a number of drugs in clinical use, such as tolbutamide, S-warfarin, phenytoin and hexobarbitone [11, 23, 24]. A number of other NSAIDs, such as diclofenac, ibuprofen, mefenamic acid, piroxicam and tenoxicam have also been reported to be metabolized by CYP2C9 [23–26]; however, these metabolic changes involved hydroxylation, whereas sulfoxidation was generally catalysed by CYP3A4 or the FMO [27, 28]. It has been reported that CYP2C9 and CYP3A4 catalysed the oxidation of sulphinpyrazone, a uricosuric agent, although CYP3A4 was approximately 10 times more effective than CYP2C9 [28]. The present study of the catalytic activities of isoforms produced by cDNA-expression systems also indicated the involvement of CYP3A4, but not FMO enzymes, in the metabolism of zaltoprofen. The catalytic activity of CYP3A4 on zaltoprofen was much smaller than that of CYP2C9, although the CYP3A4 content of the human liver is usually greater than the CYP2C9 content (CYP3A4/CYP2C9 ratio is between 1 and 8) [29]. With this in mind, it was initially speculated that zaltoprofen would be catalysed not only by CYP2C9 but also by CYP3A4, and that M-2 (the sulfoxide-metabolite) might be produced by CYP3A4. However, the involvement of CYP3A4 in zaltoprofen metabolism under physiological conditions may

actually be negligible, since ketoconazole, a selective inhibitor of CYP3A4, did not inhibit the metabolism of zaltoprofen by human liver microsomes. In addition, recombinant CYP2C9*1 was found to catalyse the sulfoxidation, but not the hydroxylation, of zaltoprofen. It is therefore interesting that the sulfoxidation of zaltoprofen involved CYP2C9, but not CYP3A4 or FMO. Furthermore, the present results highlight the need to verify data obtained with cDNA-expression systems with those from incubations with human liver microsomes before the identification of CYP isoforms involved in the metabolism of a drug can be confirmed.

A further main metabolite of zaltoprofen, the glucuronide, was produced by UGT2B7 and hydrolysed by β -glucuronidase. UGTs are encoded by multiple genes of at least two families, and the UGT2 family is divided into two subfamilies [13, 14]. Enzymes of the UGT2B subfamily catalyse the glucuronidation of several endogenous compounds (including bile acids, steroids, fatty acids and carboxylic acids) and exogenous substrates such as phenolic compounds and drugs [13, 14]. UGT enzymes are abundantly expressed in the liver and other organs, and their substrate specificity is generally considered to be low. UGT1A3 and UGT2B7 isoforms have been reported to be involved in the glucuronidation of NSAIDs in human liver. The present results showed that the ability of UGT2B7 to glucuronidate zaltoprofen was about seven times greater than that of UGT1A3. Therefore, zaltoprofen glucuronidation is mainly catalysed by UGT2B7, and other UGT isoforms contribute little to its metabolism under physiological conditions.

Serious adverse effects caused by drug–drug interactions are a great cause for concern, and any significant information that can be obtained from *in vitro* studies using human liver microsomes may be valuable in avoiding such incidents in the clinical setting [6, 9, 10]. Indeed, several methods of predicting clinical drug interactions have been established [9, 10]. In the present drug interaction study, the activities of CYP1A2, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 were inhibited to a negligible extent (< 15%) by zaltoprofen $5 \mu\text{g ml}^{-1}$, the maximum plasma concentration observed in humans after oral administration of an 80 mg zaltoprofen tablet. However, at this concentration, zaltoprofen inhibited CYP2C9 activity by 25.6%, whilst at 10 times the maximum plasma concentration it inhibited CYP2C9 activity by 75.3% and CYP3A4 activity by 48.8%. It was therefore considered appropriate to determine IC_{50} values for the effect of zaltoprofen on the activities of both these enzymes so that the likelihood of drug interactions could be predicted. The IC_{50} values were determined as $19.2 \mu\text{g ml}^{-1}$ ($64.4 \mu\text{M}$) for CYP2C9 and $53.9 \mu\text{g ml}^{-1}$ ($181 \mu\text{M}$) for CYP3A4. It is generally accepted that comparing the free concentration of a

drug in the plasma with its IC_{50} or K_i value will give an estimation of the likelihood of drug interactions [10]. The nonspecific binding of an enzyme inhibitor to microsomal protein must also be taken into account to avoid underestimation of the degree of inhibition. However, the IC_{50} values corrected for the nonspecific binding ratio were only slightly changed to be $15.5 \mu\text{g ml}^{-1}$ ($52.0 \mu\text{M}$) for CYP2C9 and $49.5 \mu\text{g ml}^{-1}$ ($166 \mu\text{M}$) for CYP3A4, since the binding of zaltoprofen to microsomal protein was very low. The free fraction of zaltoprofen in the plasma is only 0.4% [21], this is consistent with zaltoprofen's acidity, since acidic drugs are highly bound to plasma proteins but show low binding to microsomes [30]. At C_{max} , the concentration of free zaltoprofen in the plasma is only $0.02 \mu\text{g ml}^{-1}$ (67.0 nM). This is much lower than the total plasma concentration. Thus, the IC_{50} values corrected for nonspecific binding ratio for CYP2C9 and CYP3A4 are approximately 800 times and 2000 times higher than the amount of free zaltoprofen presented in the plasma at C_{max} , respectively, even allowing for the slight change in IC_{50} resulting from the correction for nonspecific binding. Similar results were obtained when the ratio of the areas under the plasma level *vs* time curves (AUCs) for CYP2C9 and CYP3A4 substrates in the presence and absence of zaltoprofen were calculated from the prediction method [10]. The unbound concentration of zaltoprofen in the liver was calculated to be $0.068 \mu\text{g ml}^{-1}$ and the increase in the AUC in the presence of zaltoprofen was estimated to be only 0.4% for CYP2C9 substrates and 0.1% for CYP3A4 substrates, respectively. Taken together, these findings suggest that zaltoprofen is unlikely to cause significant drug–drug interactions involving these isoforms. Indeed, there have as yet been no reports of clinically serious drug interactions with zaltoprofen; this may result from the fact that zaltoprofen is metabolized mainly by UGT2B7 rather than by CYP isoforms.

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