0022-3565/97/2823-1465$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 282, No. 3

Printed in U.S.A.
JPET 282:1465–1472, 1997

Involvement of Human CYP1A Isoenzymes in the Metabolism and Drug Interactions of Riluzole *In Vitro*[1]

GER-JAN SANDERINK, BRUNO BOURNIQUE, JEFFREY STEVENS, MARTINE PETRY and MICHEL MARTINET

Drug Metabolism and Pharmacokinetics Department, Rhône-Poulenc Rorer Research and Development, Antony (G.-J.S.) and Vitry/Alfortville (B.B., M.P., M.M.), France, and Collegeville, Pennsylvania (J.S.)

Accepted for publication May 9, 1997

ABSTRACT

Cytochrome P450 (CYP) and uridine diphosphate glucuronosyltransferase (UGT) isoenzymes involved in riluzole oxidation and glucuronidation were characterized in (1) kinetic studies with human hepatic microsomes and isoenzyme-selective probes and (2) metabolic studies with genetically expressed human CYP isoenzymes from transfected B-lymphoblastoid and yeast cells. *In vitro* incubation of [^{14}C]riluzole (15 μM) with human hepatic microsomes and NADPH or UDPGA cofactors resulted in formation of N-hydroxyriluzole (K_m = 30 μM) or an unidentified glucuroconjugate (K_m = 118 μM). Human microsomal riluzole N-hydroxylation was most strongly inhibited by the CYP1A2 inhibitor α-naphthoflavone (IC_{50} = 0.42 μM). Human CYP1A2-expressing yeast microsomes generated N-hydroxyriluzole, whereas human CYP1A1-expressing yeast microsomes generated N-hydroxyriluzole, two additional hydroxylated derivatives and an O-dealkylated derivative. CYP1A2 was the only genetically expressed human P450 isoenzyme in B-lymphoblastoid microsomes to metabolize riluzole. Riluzole glucuronidation was inhibited most potently by propofol, a substrate for the human hepatic UGT HP4 (UGT1.8/9) isoenzyme. *In vitro*, human hepatic microsomal hydroxylation of riluzole (15 μM) was weakly inhibited by amitriptyline, diclofenac, diazepam, nicergoline, clomipramine, imipramine, quinine and enoxacin ($IC_{50} \approx$ 200–500 μM) and cimetidine (IC_{50} = 940 μM). Riluzole (1 and 10 μM) produced a weak, concentration-dependent inhibition of CYP1A2 activity and showed competitive inhibition of methoxyresorufin O-demethylase. Thus, riluzole is predominantly metabolized by CYP1A2 in human hepatic microsomes to N-hydroxyriluzole; extrahepatic CYP1A1 can also be responsible for the formation of several other metabolites. Direct glucuronidation is a relatively minor metabolic route. *In vivo*, riluzole is unlikely to exhibit significant pharmacokinetic drug interaction with coadministered drugs that undergo phase I metabolism.

Riluzole[1] [2-amino-6-(trifluoromethoxy)benzothiazole], a novel antiglutamate agent with neuroprotective properties in animal models of neurodegenerative disease (Doble, 1996), has been shown to prolong survival in patients with ALS (Bensimon *et al.*, 1994; Lacomblez *et al.*, 1996). After oral administration to humans, the drug is almost completely absorbed, undergoes limited first-pass metabolism and is excreted predominantly *via* the urine in the form of metabolites resulting from phase I and II metabolism.[2,3]

Characterization of the CYP isoenzymes responsible for the metabolism of riluzole is of importance in assessing the likelihood of pharmacokinetic variability due to genetic polymorphism and differential regulation and in identifying potential drug interactions. In the present study, the *in vitro* oxidative metabolism and glucuronidation of riluzole were investigated using human hepatic microsomes. Identity of the CYP isoenzymes involved in riluzole biotransformation was established using genetically expressed human CYP isoenzymes from transfected cell lines and yeast and isoenzyme-selective inhibitory probes. Similarly, pathways of hepatic microsomal glucuronidation of riluzole were investigated with known inhibitors/substrates of UGT isoenzymes. To identify potential metabolic drug interactions, the effects of known CYP substrates/inhibitors and frequently coadministered drugs on the hepatic microsomal oxidation of riluzole and, conversely, the effects of riluzole on specific human hepatic CYP-dependent drug metabolism reactions were determined.

Received for publication January 30, 1997.
[1] This work was supported in part by the Bioavenir Program in conjunction with the French Ministry of Higher Education and Research.
[2] C. Gaillard, G. J. Sanderink, M. Marlard, B. Monegier, Ph. Chapelle and M. Martinet, unpublished observations.
[3] A. Le Liboux, P. Lefebvre, Y. Le Roux, P. Truffinet, M. Aubeneau, S. Kirkesseli and M. Montay: Single- and multiple-dose pharmacokinetics of riluzole in Caucasian subjects. J. Clin. Pharmacol., in press, 1997.

Materials and Methods

Chemicals

Riluzole, N-hydroxyriluzole (RPR 112512), 4-hydroxyriluzole (RP 65077), 5-hydroxyriluzole (RP 65110), 7-hydroxyriluzole (RP 65331)

ABBREVIATIONS: ALS, amyotrophic lateral sclerosis; CYP, cytochrome P450; NADPH, reduced nicotinamide adenine dinucleotide phosphate; UGT, uridine diphosphate glucuronosyltransferase; UDPGA, uridine diphosphate glucuronic acid.

and 2-amino-6-hydroxybenzothiazole (RPR 109792) were synthesized at the Centre de Recherche de Vitry/Alfortville, Rhône-Poulenc Rorer (France) and the Collegeville Chemical Processing Center, Rhône-Poulenc Rorer (Collegeville, PA). [^{14}C]Riluzole (radiochemical purity, >99%, specific activity, 56 mCi/mmol) was synthesized by the Service of Labeled Molecules of the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France). Aspirin, captopril, diazepam, enoxacin, imipramine, metronidazole, nicergoline, paracetamol, pefloxacin, ranitidine and sparfloxacin were obtained from Rhône-Poulenc Rorer. Baclofen, amitriptyline, amoxicillin, chlorpropamide, chlorzoxazone, cimetidine, clomipramine, coumarin, methoxyresorufin, nifedipine, resorufin, theophylline, thiamine, tolbutamide, troleandomycin, aniline, isoniazid, α-naphthoflavone, reduced NADPH, UDPGA, sulfaphenazole, Brij-58, 1-naphthol, 4-methylumbelliferone, lithocholic acid, bilirubin, androsterone and β-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). Furafylline and S-mephenytoin were purchased from Ultrafine Chemicals (Manchester, UK); acetanilide and quinidine sulfate were obtained from E. Merck (Darmstadt, Germany); caffeine and quinine sulfate were from Prolabo (Paris, France); SR-mephenytoin was from Sandoz (Basel, Switzerland); ketoconazole was from Biomol Research Laboratories (Plymouth Meeting, PA) and bufuralol was from Gentest Corp. (Woburn, MA). All other reagents were purchased from commercial sources and were of analytical grade.

Biological Materials

Human liver samples and preparation of microsomes. Human liver samples were obtained from male and female organ transplant donors (Eurotransplant) or from surgery (Hôpital Cochin, Paris, France). Microsomal fractions were prepared by differential ultracentrifugation. After tissue homogenization in 20 mM Tris·HCl buffer, pH 7.4, containing 0.15 M KCl, the microsomal fraction was isolated from the supernatant of a 20-min 9000 $\times$ g spin by ultracentrifugation at 105,000 $\times$ g for 60 min. The microsomal precipitate was suspended in 100 mM potassium phosphate buffer, pH 7.4, and recentrifuged at 105,000 $\times$ g for an additional 60 min. The final precipitate was resuspended in the phosphate buffer and stored at −80°C until required. A pool of human liver microsomes was also obtained from Human Biologics Inc.

Microsomes from human B-lymphoblastoid cell lines genetically engineered to express human CYP isoenzymes CYP1A1 (batch M102B), CYP1A2 (M103C), CYP2D6 (M105b), CYP2E1 (M106i) and CYP3A4 (M107d) were obtained from Gentest Corp.

Yeast cells (*Saccharomyces cerevisiae*) genetically engineered to express human CYP isoenzymes (CYP1A1, CYP1A2, CYP2C8, CYP2C9, CYP2C18, CYP3A4 and CYP3A5) and to overexpress yeast CYP450 reductase were obtained from CNRS (Gif-sur-Yvette, France) and INSERM (Paris, France), and microsomes were prepared from these cells at Rhône-Poulenc Rorer (Vitry-sur-Seine, France) as part of the Bioavenir Program. Microsomes from the same yeast strain but not expressing human CYP, were used as controls.

Microsomal Incubation

Riluzole biotransformation. Riluzole oxidation and glucuronidation were assayed by microsomal incubation of [^{14}C]-radiolabeled and unlabeled drug in the presence of the respective cofactors NADPH and UDPGA. For oxidative reactions, incubations with hepatic microsomes were performed with a suspension of hepatic microsomes (protein content, 2 mg/ml) in potassium phosphate buffer (0.1 M, pH 7.4) containing NADPH (1 mM) and $MgCl_2$ (10 mM). Lymphoblast B-cell microsomes were incubated in the same medium at a 0.5 mg/ml protein content, according to the supplier's instructions, resulting in final enzyme concentrations of 8, 18, 105, 38 and 17 pmol/ml for CYP1A1, CYP1A2, CYP2D6, CYP2E1 and CYP3A4, respectively. Yeast microsomes were incubated in Tris·HCl buffer (50 mM, pH 7.4) containing EDTA (1 mM) and NADPH (1 mM) in the absence of cytochrome b_5. For yeast microsome incubations, the final CYP450 content was 200 pmol/ml. For glucuronidation reactions, [^{14}C]riluzole (15 μM) was incubated with a suspension of hepatic microsomes (protein content, 1 mg/ml) in potassium phosphate buffer (0.1 M, pH 7.4) containing UDPGA (5 mM) and $MgCl_2$ (5 mM). For glucuronidation reactions, microsomes were activated with an optimal concentration (0.2 mg/mg of protein) of Brij-58 detergent. Incubations were performed at 25°C (yeast microsomes) or 37°C (hepatic and B-cell microsomes) in an agitating water bath, and reactions were initiated by the addition of NADPH (oxidative metabolism) or UDPGA (glucuronidation). Riluzole incubation mixtures were sampled until 20 min for monooxygenase-catalyzed reactions (30 min for expressed enzymes) and until 60 min for glucuronosyltransferase-catalyzed reactions. Reactions were terminated by the addition of an equivalent volume of methanol/acetonitrile (3.6:1 v/v) to the incubation mixture. The resulting mixture was then centrifuged at 30,000 $\times$ g for 10 min, and the supernatant was stored at 4°C before analysis.

Enzyme kinetics. Enzyme kinetic studies were performed by incubation of [^{14}C]riluzole at concentrations of 2 to 1000 μM with hepatic and CYP1A2-expressing yeast cell microsomes.

Interaction studies. In inhibition studies, hepatic microsomes were preincubated for 10 min with varying concentrations of CYP isoenzyme substrates/inhibitors (1–1000 μM) or UGT inhibitors (1–100 μM) in the presence of the appropriate cofactor (NADPH or UDPGA) before the addition of [^{14}C]riluzole (15 μM). Specific CYP isoenzyme probes included α-naphthoflavone, acetanilide and caffeine for CYP1A (Birkett *et al.*, 1993; Gonzalez, 1992), tolbutamide and sulfaphenazole for CYP2C8/9 (Birkett *et al.*, 1993), omeprazole and mephenytoin for CYP2C19 (Andersson *et al.*, 1993; Birkett *et al.*, 1993), quinidine for CYP2D6, with quinine as a negative control (Birkett *et al.*, 1993; Gonzalez, 1992), aniline, *p*-nitrophenol, chlorzoxazone and isoniazid for CYP2E1 (Birkett *et al.*, 1993; Zand *et al.*, 1993) and ketoconazole and troleandomycin for CYP3A (Back *et al.*, 1989; Birkett *et al.*, 1993). UGT inhibitors included propofol, 1-naphthol, 4-methylumbelliferone, lithocholic acid, bilirubin, androsterone, estradiol and *p*-nitrophenol.

Metabolic drug/drug interactions. In drug interaction studies with riluzole used as the substrate, hepatic microsomes were preincubated for 10 min with clomipramine, diclofenac, amitriptyline, imipramine, enoxacin, quinine, theophylline, cimetidine, caffeine, ranitidine, paracetamol, pyridoxine, enalapril, thiamine, captopril, pefloxacin, aspirin, amoxicillin, metronidazole, piracetam, baclofen, nicergoline or diazepam at concentrations ranging from 2 to 1000 μM in the presence of NADPH before the addition of [^{14}C]riluzole (15 μM).

Incubations were carried out in triplicate for experiments at single inhibitor concentrations; for determination of IC_{50} values, single incubations were performed at multiple inhibitor concentrations. K_i determinations were performed in duplicate.

Effects of riluzole on marker enzyme activities. In an additional series of drug interaction studies, the inhibitory effects of riluzole were determined on the following CYP-selective oxidative reactions: nifedipine dehydrogenation (a marker for CYP3A4) (Guengerich *et al.*, 1986); chlorzoxazone-6-hydroxylation (a marker for CYP2E1) (Peter *et al.*, 1991); bufuralol-1-hydroxylation (a marker for CYP2D6) (Kronbach *et al.*, 1987); S-mephenytoin-4-hydroxylation (a marker for CYP2C19) (Meier *et al.*, 1985; Wrighton *et al.*, 1993); tolbutamide-4-hydroxylation (a marker for CYP2C9) (Knodell *et al.*, 1987; Veronese *et al.*, 1991); coumarin-7-hydroxylation (a marker for CYP2A6) (Pearce *et al.*, 1992; Yun *et al.*, 1991); and phenacetin O-deethylation (a marker for CYP1A2) (Distlerath *et al.*, 1985; Sattler *et al.*, 1992). Human hepatic microsomes were incubated with riluzole (1 and 10 μM), and the various enzyme substrates at concentrations approximating or in excess of published K_m values. Parallel experiments were conducted with two human liver samples, and for each assay, analyses were performed in duplicate or triplicate with a NADPH- or glucose-6-phosphate-free control to quantify nonenzymatic drug metabolism. All incubation mixtures were analyzed by high-performance liquid chromatography.

The kinetic methoxyresorufin O-demethylation assay was performed on 96-well microplates in 200 μl of potassium phosphate buffer (75 mM, pH 7.64), 9 mM KCl and 1.4 mM NADH at 37°C with 0.2 mg/ml human liver microsomes or 6.6 pmol/ml yeast-expressed CYP1A2. Methoxyresorufin dissolved in DMSO and riluzole dissolved in methanol were added together to the incubation mixture, and the reaction was initiated by the addition of 500 μM NADPH (final concentration). The final incubation mixture contained 0.5% (v/v) of both solvents. Methoxyresorufin concentrations were 0.1, 0.3, 0.5, 2 and 5 μM. For each methoxyresorufin concentration, riluzole concentrations of 0, 3, 15, 30, 150 and 300 μM were tested. Resorufin production was monitored continuously with fluorescence detection (excitation wavelength, 544 nm; emission wavelength, 590 nm) over 10 min using a LabSystems Fluoroscan II microplate spectrofluorometer controlled by Biolise software. Concentrations were calculated from a resorufin standard curve.

Sample Analysis

Analysis of riluzole metabolites was carried out by high-performance liquid chromatography with a Kontron 360 automatic sampler, a 420 solvent delivery pump, a Kontron 430 UV detector (265 nm) and a Berthold LB507A radiodetector equipped with a 500-μl flow cell. The system was controlled by a Kontron MT2 Datasystem. Separation was achieved on a Lichrocart 125 × 4-mm column with a Lichrocart 4 × 4-mm guard column, both packed with Lichrosphere 60 RP Select B 5-μm particles (Merck Clevenot). The mobile phase consisted of 10 mM K_2HPO_4/methanol/acetonitrile/glacial acetic acid (108:72:20:1 v/v/v/v), eluting at a flow rate of 1 ml/min. The flow rate of the scintillation fluid was 3 ml/min, and the efficiency of the radiodetector cell was 77%.

Standard riluzole samples were prepared in phosphate buffer and mixed with methanol/acetonitrile as for the incubation samples. UV

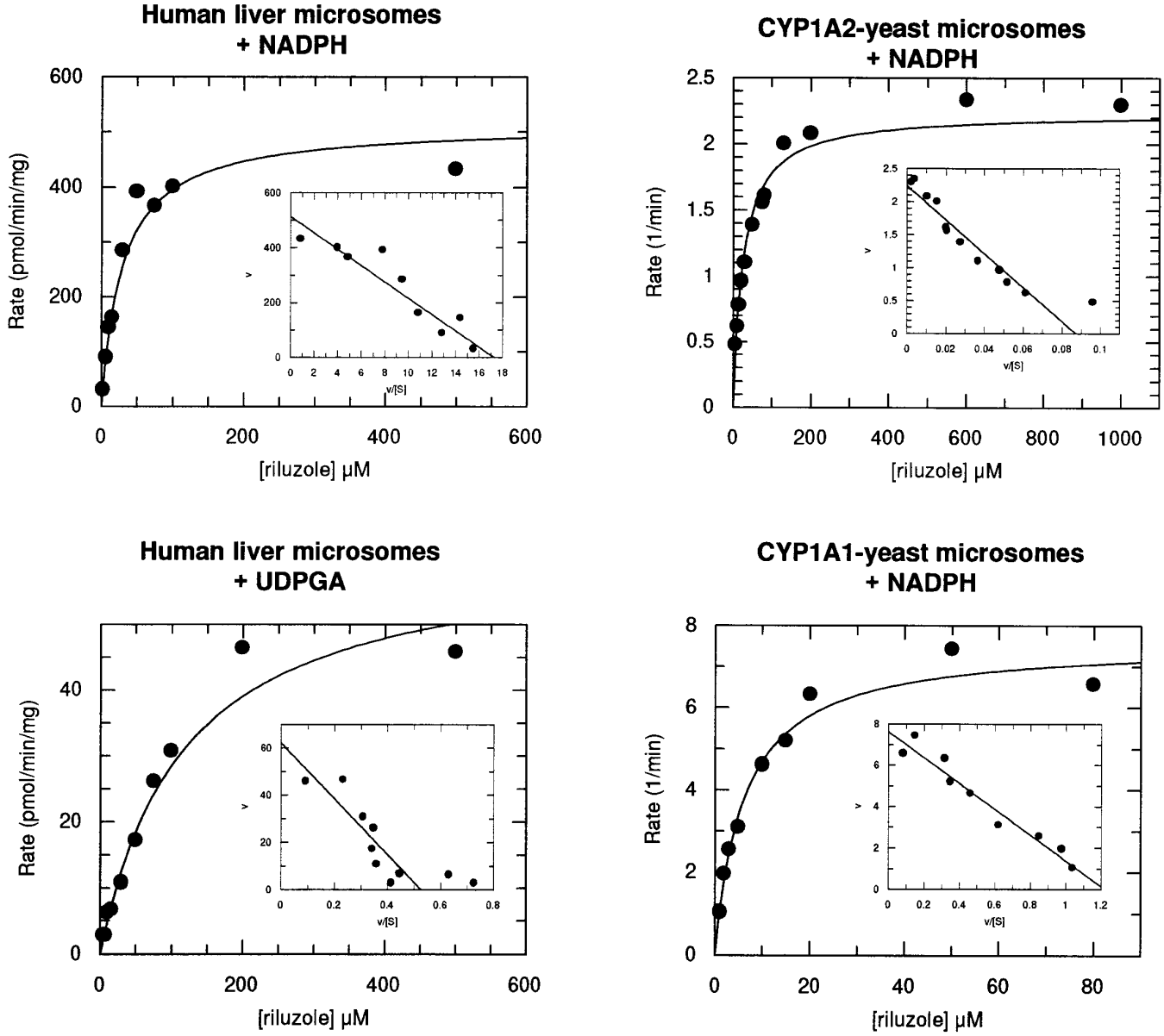

Fig. 1. Enzyme kinetics of riluzole N-hydroxylation and glucuronidation by human hepatic microsomes and by yeast microsomes expressing human CYP1A isoenzymes. Top left, saturation curve for riluzole hydroxylation by human hepatic microsomes. Top right, saturation curve for riluzole hydroxylation by yeast microsomes expressing human CYP1A2. Bottom right, saturation curve for riluzole hydroxylation by yeast microsomes expressing human CYP1A1. Bottom left, saturation curve for riluzole glucuronidation by human hepatic microsomes. Insets, Eadie-Hofstee transformations of the same data: saturation functions were obtained by fitting untransformed data to a simple Michaelis-Menten function using iterative nonlinear regression analysis.

detection of riluzole and its metabolites was linear over the concentration range of 1 to 1000 μM. The radiodetector, which was calibrated by comparing [^{14}C]riluzole peak areas with radioactivity counts in a Beckman LS 6000SC liquid scintillation counter, yielded linear detection over a concentration range of 1.35 to 500 μM.

Data Analysis

The kinetic parameters of riluzole metabolism [V_{max}, apparent K_m, K_i and IC_{50} (defined as the inhibitor concentration reducing riluzole hydroxylation by 50%)] were calculated by iterative nonlinear regression analysis using GraFit Version 3.0 software. Intrinsic metabolic clearance (Cl_m) was calculated as V_{max}/K_m. Results are expressed as mean with S.E.M.

Results

Riluzole Monooxidation

Enzyme kinetics. [^{14}C]Riluzole was metabolized in an NADPH-dependent (monooxygenase-catalyzed) manner on incubation with human hepatic microsomes, resulting in the formation of the N-hydroxylated derivative. The mean rate of riluzole N-hydroxylation by hepatic microsomes from 6 individuals was 138 ± 53 pmol/min/mg. The rate increased linearly with microsomal protein concentration up to 2.5 mg/ml. The reaction followed normal single-enzyme Michaelis-Menten kinetics (fig. 1); apparent kinetic parameters V_{max} and K_m for metabolite formation are shown in table 1.

Isoenzymes involved in phase I biotransformation of riluzole. A number of isoenzyme-selective substrates and inhibitors were screened for their ability to inhibit N-hydroxylation of riluzole by hepatic microsomes (table 2). Riluzole N-hydroxylation was markedly reduced by pretreatment with the CYP1A inhibitor α-naphthoflavone (80% inhibition at 1 μM). Consecutive experiments over a range of concentrations showed that α-naphthoflavone inhibited riluzole N-hydroxylation with an IC_{50} value of 0.42 μM. The CYP1A2 substrates caffeine (37% inhibition at 1 mM) and acetanilide (21% inhibition at 1 mM) produced a less-marked inhibition. The CYP2E1 inhibitor chlorzoxazone also weakly inhibited riluzole N-hydroxylation (36% inhibition at 100 μM; IC_{50} = 287 μM), but other inhibitors of this isoenzyme, such as aniline, isoniazid and *p*-nitrophenol, had minimal effect. Some inhibition of riluzole N-hydroxylation was observed with the CYP2C19 substrate omeprazole (31% inhibition at 100 μM) and with the CYP2D6 substrate quinidine (20% inhibition at 5 μM) but also with the negative control, quinine (table 2). Tolbutamide (a CYP2C8/9 substrate), sulfaphenazole (a CYP2C9 inhibitor), mephenytoin (a CYP2C19 substrate), ketoconazole and troleandomycin (CYP3A4 inhibitors) had no appreciable effect on riluzole N-hydroxylation.

TABLE 2

Inhibition of hepatic microsomal oxidation of riluzole by specific substrates/inhibitors of cytochrome P450 isoenzymes

Cytochrome P450 isoenzyme	Substrate/inhibitor	Concentration	Inhibition	
			Riluzole biotransformation	N-Hydroxylation
		μM	%	
CYP1A1/2	α-Naphthoflavone	1	80	72
	Caffeine	400	13	11
	Caffeine	1000	37	25
	Acetanilide	1000	21	18
CYP3A4	Ketoconazole	1	2	1
	Troleandomycin	300	12	17
CYP2D6	Quinidine	5	20	14
	Quinine (control)	5	19	13
CYP2E1	Chlorzoxazone	100	36	28
	p-Nitrophenol	100	17	15
	Aniline	50	2	8
CYP2C8/9	Tolbutamide	50	5	1
CYP2C19	Omeprazole	100	31	24

On incubation of [^{14}C]riluzole with NADPH and microsomes from human cytochrome P450-expressing B-lymphoblastoid cells, N-hydroxylation was confined to those containing CYP1A2, with no metabolism occurring with CYP1A1-, CYP2D6-, CYP2E1- or CYP3A4-containing microsomes or control microsomes. In the case of human cytochrome P450-expressing yeast cells, microsomes containing CYP1A2 generated N-hydroxyriluzole on incubation with [^{14}C]riluzole, whereas microsomes containing CYP1A1 gave rise to the hydroxylated derivatives N-hydroxyriluzole, 4-hydroxyriluzole and 5-hydroxyriluzole; the O-dealkylated derivative 2-amino-6-hydroxybenzothiazole; and, to a lesser extent, 7-hydroxyriluzole (fig. 2). Maximum riluzole biotransformation rates with CYP1A2- and CYP1A1-containing yeast microsomes were 2.24 and 7.61 pmol/min/pmol of P450, with a K_m value of 25.7 and 6.2 μM, respectively. Yeast microsomes containing CYP3A4, CYP3A5, CYP2C8, CYP2C9 or CYP2C18 and control microsomes did not produce any detectable metabolite.

Effects of drugs on riluzole biotransformation. Of the drugs screened for their effect on riluzole N-hydroxylation by human hepatic microsomes, the most potent inhibitors were amitriptyline, clomipramine, diazepam, diclofenac, and nicergoline, with IC_{50} values of 210 to 260 μM (table 3). Enoxacin,

TABLE 1

Michaelis-Menten kinetic parameters for riluzole biotransformation by human hepatic microsomes and CYP1A-expressing yeast microsomes

Enzyme source	Cosubstrate	V_{max}	K_m	Cl_{int}
			μM	
Hepatic microsomes	NADPH	513 ± 36[a]	29.7 ± 3.5	17.28[c]
CYP1A2-yeast microsomes	NADPH	2.24 ± 0.12[b]	25.7 ± 2.2	0.09[d]
CYP1A1-yeast microsomes	NADPH	7.61 ± 0.34[b]	6.24 ± 0.55	1.22[d]
Hepatic microsomes	UDPGA	62.0 ± 10.5[a]	118 ± 27	0.53[c]

Cl_{int} = intrinsic metabolic clearance.
[a] pmol/min/mg.
[b] min^{-1}.
[c] μl/min/mg.
[d] μl/min/pmol.
Values are mean ± S.E.M.

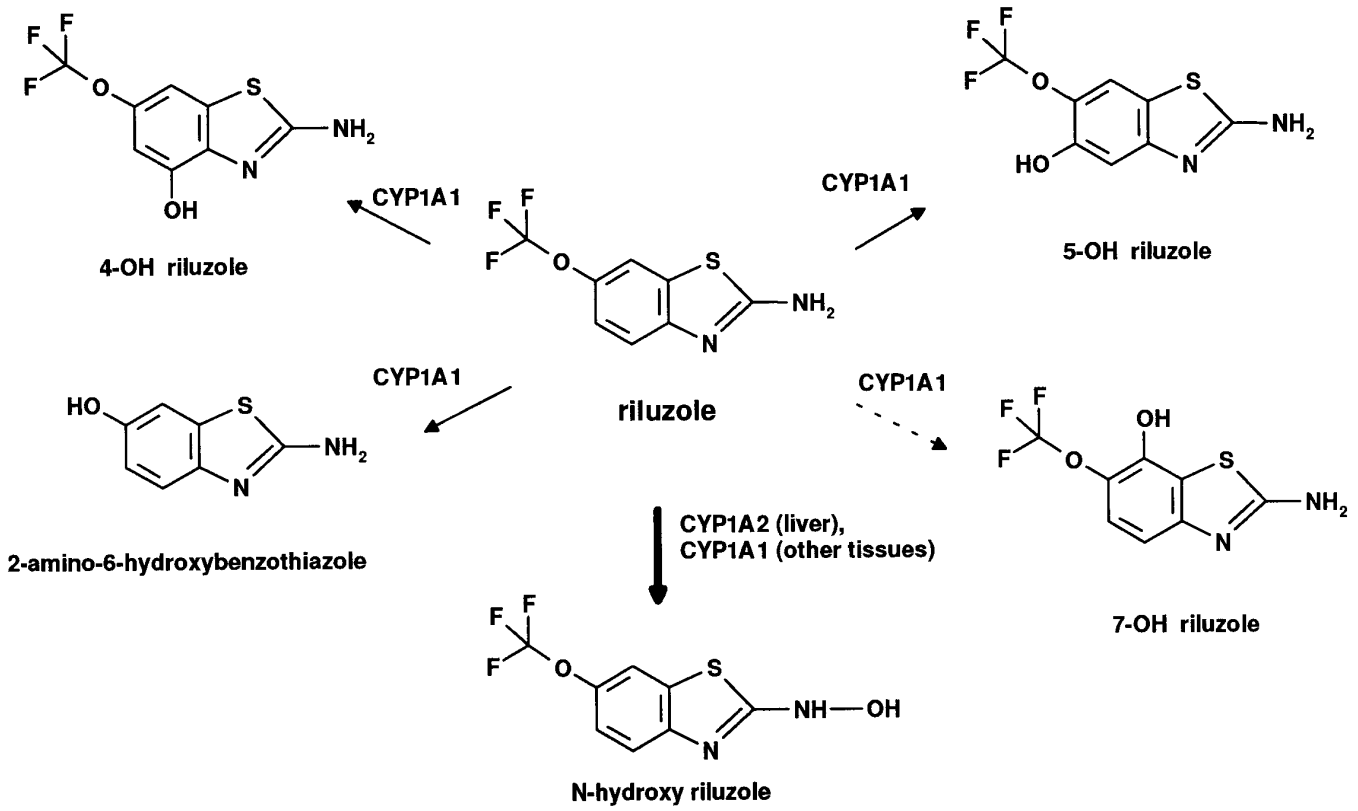

Fig. 2. Metabolic pathways involved in phase I biotransformation of riluzole.

TABLE 3

Effects of coadministered drugs on *in vitro* N-hydroxylation of riluzole by human hepatic microsomes

Drug	Inhibition[a]	IC_{50}
	%	μM
Clomipramine	99	265 ± 25
Diclofenac	99	234 ± 19
Amitriptyline	94	210 ± 17
Imipramine	85	356 ± 20
Enoxacin	71	465 ± 77
Nicergoline	71	256 ± 13
Quinine	63	444 ± 62
Diazepam	59	240 ± 23
Theophylline	47	
Cimetidine	40	937 ± 182
Caffeine	37	
Ranitidine	29	
Paracetamol	28	
Pyridoxine	18	
Enalapril	12	

[a] Inhibition measured at a riluzole concentration of 15 μM and an inhibitor concentration of 1 mM for all drugs except nicergoline and diazepam (both 0.5 mM).

imipramine and quinine (all at 1 mM) also caused >50% inhibition of riluzole N-hydroxylation. Intermediate inhibition (25–50%) was observed with theophylline, cimetidine, caffeine, ranitidine and paracetamol, whereas minimal inhibition (<10%) was seen with thiamine, captopril, pefloxacin, aspirin, amoxicillin, metronidazole, piracetam and baclofen at concentrations of 1 mM. Sparfloxacin (≥400 μM) had no effect on parent riluzole biotransformation but did reduce levels of N-hydroxyriluzole, suggesting that it may react directly with this metabolite rather than with the parent compound.

Effect of riluzole on P450 enzyme activities. Riluzole at 1 and 10 μM had a weak inhibitory effect on human hepatic microsomal CYP1A2-mediated phenacetin O-deethylation, CYP2A6-mediated coumarin-7-hydroxylation, CYP2D6-mediated bufuralol-1-hydroxylation and CYP2E1-mediated chlorzoxazone-6-hydroxylation (table 4). Only the effects on phenacetin O-deethylation and chlorzoxazone-6-hydroxylation were concentration dependent. Because riluzole is metabolized by the CYP1A2 isoenzyme, we studied the inhibition kinetics of CYP1A2-catalyzed methoxyresorufin-O-demethylation. Riluzole competitively inhibited methoxyresorufin-O-demethylation with an inhibition constant (K_i) of 12.1 ± 1.5 μM in human liver microsomes and 16.7 ± 1.4 μM in microsomes from CYP1A2-expressing yeast (fig. 3). No appreciable or consistent inhibition of CYP2C9-catalyzed tolbutamide 4-hydroxylation, CYP2C19-catalyzed S-mephenytoin 4-hydroxylation or CYP3A4-catalyzed nifedipine dehydrogenation was seen with riluzole.

Riluzole Glucuronidation

Enzyme kinetics. Riluzole was metabolised in an UDPGA-dependent (*i.e.*, UGT catalyzed) manner on incubation with detergent-activated human hepatic microsomes, resulting in the formation of a single unidentified metabolite. The reaction followed normal single-enzyme Michaelis-Menten kinetics (fig. 1); apparent Michaelis-Menten kinetic parameters V_{max} and K_m are shown in table 1.

Inhibition of riluzole glucuronidation

Riluzole glucuronidation was inhibited in a concentration-dependent manner by preincubation with propofol (IC_{50} = 18.7 μM), indicating the involvement of the UGT HP4 (UGT1.8/9)

TABLE 4
Effects of riluzole (1 and 10 μM) on human hepatic cytochrome P450-mediated oxidative drug metabolism

Drug oxidation	P450 isoenzyme	Change in drug metabolism			
		Low substrate concentration[a]		High substrate concentration[a]	
		1 μM riluzole	10 μM riluzole	1 μM riluzole	10 μM riluzole
		%			
Phenacetin-O-deethylation	CYP1A2	+5	−19	0	−12
Coumarin-7-hydroxylation	CYP2A6	−13	−17	−2	−2
Tolbutamide-4-hydroxylation	CYP2C9	+6	+3	+10	+4
S-Mephenytoin-4-hydroxylation	CYP2C19	+1	−2	−1	+3
Bufuralol-1-hydroxylation	CYP2D6	−9	−14	−11	−14
Chlorzoxazone-6-hydroxylation	CYP2E1	−5	−28	−7	−20
Nifedipine dehydrogenation	CYP3A4	−15	+4	−4	+8

[a] Substrate concentrations: nifedipine, 25 and 50 μM; chlorzoxazone, 40 and 80 μM; bufuralol, 15 and 30 μM; S-mephenytoin, 100 and 200 μM; tolbutamide, 125 and 250 μM; coumarin, 0.5 and 1 μM; phenacetin, 40 and 100 μM.
+, Enhancement of drug metabolism; −, inhibition of drug metabolism.
Results are expressed as the mean values for two human liver samples.

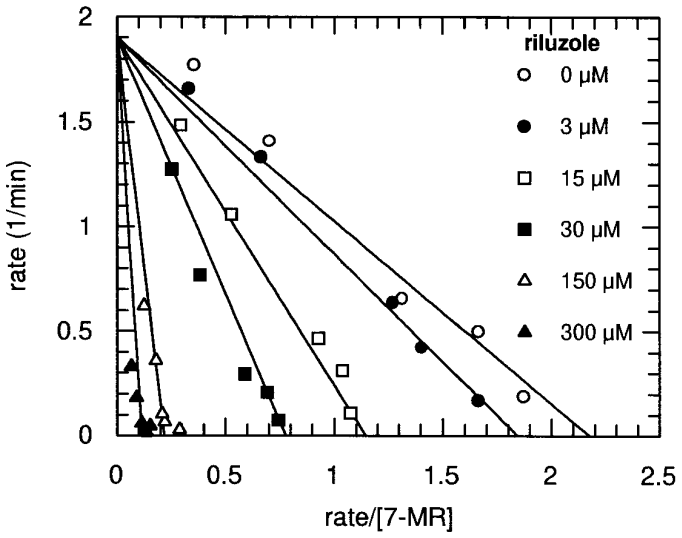

Fig. 3. Inhibition by riluzole of methoxyresorufin O-demethylation by yeast microsomes expressing human CYP1A2. Data are presented as Eadie-Hofstee transformations of saturation curves obtained in the presence of various concentrations of riluzole; saturation functions were obtained by fitting untransformed data to a simple Michaelis-Menten function using iterative nonlinear regression analysis.

isoenzyme in this reaction (Ebner and Burchell, 1993). Maximum inhibition (70%) was seen with propofol 100 μM, whereas less-marked inhibition was obtained with 100 μM estradiol (40% inhibition), 100 μM androsterone (38% inhibition), 100 μM lithocholic acid (37% inhibition), 50 μM bilirubin (28% inhibition) and 100 μM *p*-nitrophenol (23% inhibition).

Discussion

Two types of human hepatic enzyme are involved in the biotransformation of riluzole by the human liver *in vitro*: monooxygenases and UDP-glucuronosyltransferases. Quantitatively, monooxygenation is possibly the more important reaction because intrinsic clearance *via* this route was 30-fold higher than that *via* direct glucuronidation.

Riluzole N-hydroxylation was the only monooxygenase-mediated reaction observed with human hepatic microsomes. CYP1A2 appears to be the main isoenzyme involved in this reaction, a conclusion that is based on the potent inhibition observed with the specific CYP1A2 inhibitor α-naphthoflavone (IC_{50} = 0.42 μM), the specific biotransformation noted with genetically expressed CYP1A2, and the competitive inhibitory effect of riluzole on CYP1A2-catalyzed methoxyresorufin O-demethylation. The relatively weak inhibitory effect of the CYP1A2 substrates caffeine and acetanilide on riluzole N-hydroxylation may be attributed to the low affinity of these compounds for CYP1A2 (K_m = 0.5 to 1.5 mM) (Grant *et al.*, 1987) compared with that of riluzole (K_m = 23 μM). In keeping with the present finding, the CYP1A2 isoenzyme has previously been shown to catalyze specifically the N-hydroxylation of many heterocyclic (aryl) amines in humans (Boobis *et al.*, 1994; Gonzalez and Idle, 1994). Because CYP1A2 seems to be the only CYP1A isoenzyme expressed in human liver (Gonzalez, 1992), it can be concluded that this is the major isoenzyme involved in the hepatic metabolism of riluzole. CYP1A isoenzymes are readily induced *in vivo* by tobacco smoke (Guengerich and Shimada, 1991). Interestingly, increased riluzole clearance in smokers has been demonstrated in a recent population pharmacokinetic study of riluzole.[4]

Possible extrahepatic metabolism of riluzole is suggested by the finding that microsomes of genetically engineered yeast cells expressing human CYP1A1 catalyzed the formation of several hydroxylated derivatives that have previously been identified in the urine of patients treated with riluzole.[2] The CYP1A1 isoenzyme is expressed largely in extrahepatic tissue, such as the lung (Gonzalez, 1992).

Although the disparate effects of the various CYP2E1 substrates on riluzole N-hydroxylation appear somewhat contradictory (some inhibition occurring with chlorzoxazone, but not with aniline, isoniazid or *p*-nitrophenol), it should be noted that not all these substrates are highly specific for CYP2E1. Thus, although chlorzoxazone and *p*-nitrophenol share similarly high affinities ($K_m \approx$ 30 μM) for CYP2E1 (Peter *et al.*, 1991; Tassaneeyakul *et al.*, 1993a), chlorzoxazone is also metabolized by CYP1A2 (Ono *et al.*, 1996). The lack of inhibitory effect of *p*-nitrophenol at concentrations as high as 1 mM and the absence of metabolism by CYP2E1 expressed in B-lymphoblastoid cells suggest that this isoenzyme does not play an appreciable role in riluzole oxidation.

[4] R. Bruno, N. Vivier, G. Montay, A. Le Liboux, L. K. Powe, J. C. Delumeau and G. R. Rhodes. Population pharmacokinetics of riluzole in patients with amyotrophic lateral sclerosis. Clin. Pharmacol. Ther., in press, 1997.

The use of *in vitro* systems such as human hepatic microsomes in drug-interaction studies is recommended for predicting the consequences of concurrent drug therapy (Peck *et al.*, 1993). As a substrate for specific P450 isoforms, riluzole has the potential to act as a competitive enzyme inhibitor and thereby alter the metabolism and pharmacokinetics of coadministered drugs that are also subject to phase I metabolism. Effectively, riluzole is a competitive inhibitor of CYP1A2-catalyzed methoxyresorufin O-demethylation, with a K_i value close to its K_m value. At *in vitro* concentrations of 1 and 10 μM, similar to or higher than those achieved therapeutically,[3] riluzole had a weak inhibitory effect on human hepatic microsomal CYP1A2-, CYP2A6-, CYP2D6- and CYP2E1-mediated oxidative drug metabolism. Apart from inhibition of methoxyresorufin O-demethylation, the most pronounced, concentration-dependent inhibition (28%) was that of CYP2E1-catalyzed chlorzoxazone-6-hydroxylation. However, as mentioned above, this inhibition probably reflects on CYP1A2 as well as CYP2E1. Inhibition by riluzole of microsomal CYP1A2-catalyzed phenacetin O-deethylation is not unexpected given the evidence for the involvement of this isoenzyme in riluzole metabolism. However, the weak inhibitory effect of riluzole suggests that it is unlikely to alter to any appreciable extent the hepatic clearance of drugs that are oxidized by the CYP system.

Not surprisingly, known drug substrates of CYP1A2, including enoxacin (Edwards *et al.*, 1988), cimetidine (Knodell *et al.*, 1991), paracetamol (Raucy *et al.*, 1989), imipramine (Lemoine *et al.*, 1993) and the methylxanthines caffeine and theophylline (Fuhr *et al.*, 1992; Tassaneeyakul *et al.*, 1993b), had an inhibitory effect (IC_{50} values $\geq$ 400 μM) on riluzole N-hydroxylation. In contrast to enoxacin, and in keeping with their lack of effect on theophylline metabolism *in vivo* or *in vitro* (Edwards *et al.*, 1988), the quinolones pefloxacin and sparfloxacin had no direct effect on riluzole hydroxylation. The inhibitory effect of cimetidine, a well known CYP inhibitor *in vivo* (Smith and Kendall, 1988), was comparatively weak (IC_{50} = 937 μM). However, it has previously been noted that cimetidine inhibition can be underestimated *in vitro*, possibly because its interaction with CYP proceeds rather slowly (Chang *et al.*, 1992).

Although the enzymes responsible for clomipramine and amitriptyline metabolism have not been identified, both these tricyclic antidepressants are susceptible to interaction with fluvoxamine, a potent CYP1A2 inhibitor (Berchty *et al.*, 1991; Brøsen *et al.*, 1993). Moreover, both imipramine and amitriptyline have been shown to be mechanism-based inhibitors of CYP (Murray and Field, 1992), so an effect of these drugs on riluzole metabolism is not unexpected.

For all the tested drugs, the IC_{50} value was $\geq$14 times greater than the riluzole concentration (15 μM) in the incubate. Therefore, inhibition of riluzole metabolism appears *a priori* unlikely, but results from *in vivo* drug-interaction studies are required before it can be concluded that these agents effectively inhibit riluzole metabolism or alter its pharmacokinetics in humans.

Knowledge of UGT isoenzymes and their substrate specificity is much more limited than is the case with the CYP system. Nevertheless, using genetically expressed enzymes, Ebner and Burchell (1993) established that within the *UGT1* gene family, propofol is a specific substrate for UGT HP4 (UGT1.8/9), whereas 1-naphthol is more specific for UGT HP1 and bilirubin is specific for UGT HP2 and UGT HP3. Therefore, in view of the pronounced inhibitory effect of propofol and the minimal effect of 1-naphthol and bilirubin, riluzole conjugation is most likely to be mediated by the UGT HP4 isoenzyme. Although several other compounds, many of them substrates for UGT2 isoenzymes (androsterone, estradiol, lithocholic acid), inhibited riluzole glucuronidation to varying extents, lack of substrate specificity and the low biotransformation rate of riluzole make it difficult to evaluate the significance of these findings.

In conclusion, riluzole is predominantly metabolized by CYP1A2 in human hepatic microsomes, whereas extrahepatic CYP1A1 is also responsible for the formation of several human metabolites that are also observed *in vivo*. The fact that riluzole is a specific substrate for the CYP1A2 isoenzyme, has a single oxidative metabolic pathway in the liver and is a nontoxic drug with low metabolic clearance in humans could make it an interesting candidate as an *in vitro* and *in vivo* probe. This is further demonstrated by the effect of tobacco use on riluzole clearance in patients with ALS. Direct glucuronidation is a relatively minor metabolic route and is catalyzed by UGT HP4. On the basis of *in vitro* findings, at therapeutic doses riluzole is unlikely to alter the pharmacokinetics of coadministered drugs that undergo phase I metabolism. Conversely, significant modification of the pharmacokinetics of riluzole by these drugs would not be anticipated in clinical practice, although this has yet to be confirmed.

Acknowledgments

The authors thank Ms. Annick Touzet, Ms. Helene Heyn, Ms. Shamsi Raeissi, Mr. Zuyu Guo and Mr. Rachid Boukaiba for their excellent technical assistance and Dr. Adam Doble for useful discussions regarding the manuscript.

References

Andersson, T., Miners, J. O., Veronese, M. E., Tassaneeyakul, W., Tassaneeyakul, W., Meyer, U. A. and Birkett, D. J.: Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. Br. J. Clin. Pharmacol. **36:** 521–530, 1993.

Back, D. J., Stevenson, P. and Tjia, J. F.: Comparative effects of two antimycotic agents, ketoconazole and terbinafine on the metabolism of tolbutamide, ethinyloestradiol, cyclosporin and ethoxycoumarin by human liver microsomes *in vitro*. Br. J. Clin. Pharmacol. **28:** 166–170, 1989.

Bensimon, G., Lacomblez, L., Meininger, V. and the ALS/Riluzole Study Group: A controlled trial of riluzole in amyotrophic lateral sclerosis. N. Engl. J. Med. **330:** 585–591, 1994.

Berchty, G., Vandel, S., Vandel, B., Allers, G. and Volmat, R.: Fluvoxamine-tricyclic antidepressant interaction. Eur. J. Clin. Pharmacol. **40:** 119–120, 1991.

Birkett, D. J., Mackenzie, P. I., Veronese, M. E. and Miners, J. O.: *In vitro* approaches can predict human drug metabolism. Trends Pharmacol. Sci. **14:** 292–294, 1993.

Boobis, A. R., Lynch, A. M., Murray, S., De la Torre, R., Solans, A., Farre, M., Segura, J., Gooderham, N. J. and Davies, D. S.: CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. Cancer Res. **54:** 89–94, 1994.

Brøsen, K., Skjelbo, E., Rasmussen, B. B., Polsen, H. E. and Loft, S.: Fluvoxamine is a potent inhibitor of cytochrome P4501A2. Biochem. Pharmacol. **45:** 1211–1214, 1993.

Chang, T., Levine, M. and Bellward, G. D.: Selective inhibition of rat hepatic microsomal cytochrome P-450. II. Effect of *in vitro* administration of cimetidine. J. Pharmacol. Exp. Ther. **260:** 1450–1455, 1992.

Distlerath, L., Reilly, P., Martin, M., Davis G. C., Wilkinson, G. R. and Guengerich, F. P.: Purification and characterization of the human liver cytochromes P450 involved in debrisoquine 4-hydroxylation and phenacetin *O*-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. J. Biol. Chem. **260:** 9057–9067, 1985.

Doble, A.: The pharmacology and mechanism of action of riluzole. Neurology **47:** S233–S241, 1996.

Ebner, T. and Burchell, B.: Substrate specificities of two stably expressed

human liver UDP-glucuronosyltransferases of the UGT1 gene family. Drug. Metab. Dispos. **21:** 50–55, 1993.
EDWARDS, D. J., BOWLES, S. K., SVENSSON, C. K. AND RYBAK, M. J.: Inhibition of drug metabolism by quinolone antibiotics. Clin. Pharmacokinet. **15:** 194–204, 1988.
FUHR, U., DOEHMER, J., BATTULA, N., WOELFEL, C., KUDLA, C. AND KEITA, Y.: Biotransformation of caffeine and theophylline in mammalian cell lines genetically engineered for expression of single cytochrome P450 isoforms. Biochem. Pharmacol. **43:** 225–235, 1992.
GONZALEZ, F. J.: Human cytochromes P450: Problems and prospects. Trends Pharmacol. Sci. **13:** 346–352, 1992.
GONZALEZ, F. J. AND IDLE, J. R.: Pharmacogenetic phenotyping and genotyping. Clin. Pharmacokinet. **26:** 59–70, 1994.
GRANT, D. M., CAMPBELL, M. E., TANG, B. K. AND KALOW, W.: Biotransformation of caffeine by microsomes from human liver. Biochem. Pharmacol. **36:** 1251–1260, 1987.
GUENGERICH, F. P., MARTIN, M., BEAUNE, P., KREMERS, P., WOLFF, T. AND WAXMAN, D. J.: Characterization of rat and human liver microsomal cytochrome P450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. J. Biol. Chem. **261:** 5051–5060, 1986.
GUENGERICH, F. P. AND SHIMADA, T.: Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. Chem. Res. Toxicol. **4:** 391–407, 1991.
KNODELL, R. G., BROWNE, D. G., GWOZDZ, G. P., BRIAN, W. R. AND GUENGERICH, F. P.: Differential inhibition of individual human liver cytochromes P-450 by cimetidine. Gastroenterology **101:** 1680–1691, 1991.
KNODELL, R. G., HALL, S. D., WILKINSON, G. R. AND GUENGERICH, F. P.: Hepatic metabolism of tolbutamide: Characterization of the form of cytochrome P450 involved in methyl hydroxylation and relationship to *in vivo* disposition. J. Pharmacol. Exp. Ther. **241:** 1112–1119, 1987.
KRONBACH, T., MATHYS, D., GUT, J., CATIN, T. AND MEYER, U.: High performance liquid chromatographic assays for bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase and dextromethorphan *O*-demethylase in microsomes and purified cytochrome P450 isozymes of human liver. Anal. Biochem. **162:** 24–32, 1987.
LACOMBLEZ, L., BENSIMON, G., LEIGH, P. N., GUILLET, P. AND MEININGER, V., for the Amyotrophic Lateral Sclerosis/Riluzole Study Group II: Dose-ranging study of riluzole in amyotrophic lateral sclerosis. Lancet **347:** 1425–1431, 1996.
LEMOINE, A., GAUTIER, J. C., AZOULAY, D., KIFFEL, L., BELLOC, C., GUENGERICH, F. P., MAUREL, P., BEAUNE, P. AND LEROUX, J. P.: Major pathway of imipramine metabolism is catalyzed by cytochromes P-450 1A2 and P-450 3A4 in human liver. Mol. Pharmacol. **43:** 827–832, 1993.
MEIER, U. T., KRONBACH, T. AND MEYER, U. A.: Assay of mephenytoin metabolism in human liver microsomes by high-performance liquid chromatography. Anal. Biochem. **151:** 286–291, 1985.
MURRAY, M. AND FIELD, S. L.: Inhibition and metabolite complexation of rat hepatic microsomal cytochrome P450 by tricyclic antidepressants. Biochem. Pharmacol **43:** 2065–2071, 1992.
ONO, S., HATANAKA, T., HOTTA, H., SATOH, T., GONZALEZ, F. J. AND TSUTSUI, M.: Specificity of substrate and inhibitor probes for cytochrome P450s: Evaluation of in vitro metabolism using cDNA-expressed human P450s and human liver microsomes. Xenobiotica **26:** 681–693, 1996.
PEARCE, R., GREENWAY, D. AND PARKINSON, A.: Species differences and interindividual variation in liver microsomal cytochrome P450 2A enzymes: Effects on coumarin, dicumarol and testosterone oxidation. Arch. Biochem. Biophys. **298:** 211–225, 1992.
PECK, C., TEMPLE, R. AND COLLINS, J.: Understanding the consequences of concurrent therapies. JAMA **269:** 1550–1552, 1993.
PETER, R., BOCKER, R., BEAUNE, P. H., IWASAKI, M., GUENGERICH, F. P. AND YANG, C. S.: Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P450llE1. Chem. Res. Toxicol. **3:** 566–576, 1991.
RAUCY, J. L., LASKER, J. M., LIEBER, C. S. AND BLACK, M.: Acetaminophen activation by human liver microsomes P450llE1 and P450lA2. Arch. Biochem. Biophys. **271:** 270–283, 1989.
SATTLER, M., GUENGERICH, F. P., YUN, C. H., CHRISTIANS, U. AND SEWING, K. F.: Cytochrome P450 3A enzymes are responsible for biotransformation of FK506 and rapamycin in man and rat. Drug Metab. Dispos. **20:** 753–761, 1992.
SMITH, S. R. AND KENDALL, M. J.: Ranitidine versus cimetidine: A comparison of their potential to cause clinically important drug interactions. Clin. Pharmacokinet. **15:** 44–56, 1988.
TASSANEEYAKUL, W., VERONESE, M. E., BIRKETT, D. J., GONZALEZ, F. J. AND MINERS, J. O.: Validation of 4-nitrophenol as an *in vitro* substrate probe for human liver CYP2E1 using cDNA expression and microsomal kinetic techniques. Biochem. Pharmacol. **46:** 1975–1981, 1993a.
TASSANEEYAKUL, W., BIRKETT, D. J., VERONESE, M. E., MCMANUS, M. E., TUKEY, R. H., QUATTROCHI, L. C., GELBOIN, H. V. AND MINERS, J. O.: Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. J. Pharmacol. Exp. Ther. **265:** 401–407, 1993b.
VERONESE, M., MACKENZIE, P. I., DOECKE, C., MCMANUS M. E., MINERS, J. O. AND BIRKETT, D. J.: Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. Biochem. Biophys. Res. Commun. **175:** 1112–1118, 1991.
WRIGHTON, S., STEVENS, J., BECKER, G. AND VANDENBRANDEN, M.: Isolation and characterization of human liver cytochrome P450 2C19: Correlation between 2C19 and S-mephenytoin 4′-hydroxylation. Arch. Biochem. Biophys. **306:** 240–245, 1993.
YUN, C.-H., SHIMADA, T. AND GUENGERICH, F.: Purification and characterization of human liver microsomal cytochrome P450 2A6. Mol. Pharmacol. **40:** 679–685, 1991.
ZAND, R., NELSON, S. D., SLATTERY, J. T., KALHORN, T. F., ADAMS, S. P. AND WRIGHT, J. M.: Inhibition and induction of cytochrome P4502E1-catalyzed oxidation by isoniazid in humans. Clin. Pharmacol. Ther. **54:** 142–149, 1993.

Send reprint requests to: G. J. Sanderink, Ph.D., Drug Metabolism and Pharmacokinetics Department (Tri 58), Rhône-Poulenc Rorer, 20 Avenue Raymond Aron, F-92165 Antony Cedex, France. E-mail: gerard.sanderink@rhone-poulenc.com.