British Journal of Pharmacology (2003) 138, 1465–1474
www.nature.com/bjp

Contribution of human cytochrome *P*-450 isoforms to the metabolism of the simplest phenothiazine neuroleptic promazine

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1 The aim of the present study was to identify human cytochrome *P*-450 isoforms (CYPs) involved in 5-sulphoxidation and N-demethylation of the simplest phenothiazine neuroleptic promazine in human liver.

2 The experiments were performed in the following *in vitro* models: (A) a study of promazine metabolism in liver microsomes—(a) correlations between the rate of promazine metabolism and the level and activity of CYPs; (b) the effect of specific inhibitors on the rate of promazine metabolism (inhibitors: CYP1A2—furafylline, CYP2D6—quinidine, CYP2A6 + CYP2E1—diethyldithiocarbamic acid, CYP2C9—sulfaphenazole, CYP2C19—ticlopidine, CYP3A4—ketoconazole); (B) promazine biotransformation by cDNA-expressed human CYPs (Supersomes 1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2E1, 3A4); (C) promazine metabolism in a primary culture of human hepatocytes treated with specific inducers (rifampicin—CYP3A4, CYP2B6 and CYP2C inducer, 2,3,7,8-tetrachlordibenzeno-*p*-dioxin (TCDD)—CYP1A1/1A2 inducer).

3 In human liver microsomes, the formation of promazine 5-sulphoxide and *N*-desmethylpromazine was significantly correlated with the level of CYP1A2 and ethoxyresorufin *O*-deethylase and acetanilide 4-hydroxylase activities, as well as with the level of CYP3A4 and cyclosporin A oxidase activity. Moreover, the formation of N-desmethylpromazine was correlated well with *S*-mephenytoin 4′-hydroxylation.

4 Furafylline (a CYP1A2 inhibitor) and ketoconazole (a CYP3A4 inhibitor) significantly decreased the rate of promazine 5-sulphoxidation, while furafylline and ticlopidine (a CYP2C19 inhibitor) significantly decreased the rate of promazine *N*-demethylation in human liver microsomes.

5 The cDNA-expressed human CYPs generated different amounts of promazine metabolites, but the rates of CYP isoforms to catalyse promazine metabolism at therapeutic concentration (10 μM) was as follows: 1A1 > 2B6 > 1A2 > 2C9 > 3A4 > 2E1 > 2A6 > 2D6 > 2C19 for 5-sulphoxidation and 2C19 > 2B6 > 1A1 > 1A2 > 2D6 > 3A4 > 2C9 > 2E1 > 2A6 for N-demethylation. The highest intrinsic clearance (V_{max}/K_m) was found for CYP1A subfamily, CYP3A4 and CYP2B6 in the case of 5–sulphoxidation, and for CYP2C19, CYP1A subfamily and CYP2B6 in the case of *N*-demethylation.

6 In a primary culture of human hepatocytes, TCDD (a CYP1A subfamily inducer), as well as rifampicin (mainly a CYP3A4 inducer) induced the formation of promazine 5-sulphoxide and *N*-desmethylpromazine.

7 Regarding the relative expression of various CYPs in human liver, the obtained results indicate that CYP1A2 and CYP3A4 are the main isoforms responsible for 5-sulphoxidation, while CYP1A2 and CYP2C19 are the basic isoforms that catalyse *N*-demethylation of promazine in human liver. Of the other isoforms studied, CYP2C9 and CYP3A4 contribute to a lesser degree to promazine 5-sulphoxidation and N-demethylation, respectively. The role of CYP2A6, CYP2B6, CYP2D6 and CYP2E1 in the investigated metabolic pathways of promazine seems negligible.
British Journal of Pharmacology (2003) **138,** 1465 – 1474. doi:10.1038/sj.bjp.0705195

Keywords: Promazine; metabolism; human CYP; microsomes; Supersomes; hepatocytes; specific inhibitors

Abbreviations: ACT, acetanilide 4-hydroxylation; CHLRZ, chlorzoxazone 6-hydroxylation; COUM, coumarin 7-hydroxylation; CsA, cyclosporin A oxidation; CYP, cytochrome *P*-450; DDC, diethyldithiocarbamic acid; EROD, ethoxyresorufin *O*-deethylation; FURAF, furafylline; HPLC, high-performance liquid chromatography; K_m, the Michaelis constant; KET, ketoconazole; N-MEPH, *S*-mephenytoin N-demethylation; OH-MEPH, *S*-mephenytoin 4′-hydroxylation; QUIN, quinidine; SULF, sulfaphenazole; TICLOP, ticlopidine; TCDD, 2,3,7,8-tetrachlordibenzeno-*p*-dioxin; TOLB, tolbutamide 4-methylhydroxylation; V_{max}, maximum velocity of the reaction

Introduction

Among the phenothiazine neuroleptics, promazine is the weakest blocker of dopamine D_2 and serotonin 5-HT_2 receptors (Seeman *et al*., 1975; Creese *et al*., 1976; Peroutka & Snyder, 1980; Richelson & Nelson, 1984), but it is a relatively strong antagonist of adrenergic α_1 and histamine H_1 receptors (Richelson & Nelson, 1984). According to the above drug–receptor interactions, promazine is one of the mildest

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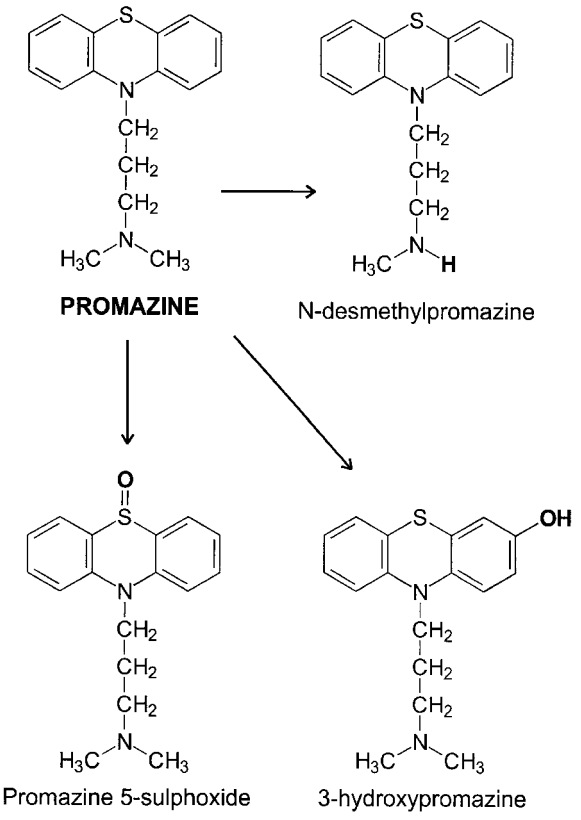

Figure 1 Metabolic pathways of promazine.

neuroleptics, displaying a relatively sedative profile of action that markedly surpasses its antischizophrenic potency. Therefore promazine is recommended for the treatment of psychoses in elderly patients, as well as for the therapy of somatogenic psychoses with the symptoms of anxiety and fear (Hu *et al*., 1990).

During phase I of metabolism, neuroleptics that are phenothiazine derivatives, for example promazine (Figure 1), undergo mainly S-oxidation in the thiazine ring in position 5 and N-demethylation in a side chain, as well as aromatic hydroxylation and N-oxidation (Svendsen & Bird, 1986). In man, N-demethylation and sulphoxidation were reported to be the dominant pathways of promazine biotransformation (Goldenberg *et al*., 1965). Similar promazine metabolism was observed in animals (Weir & Sanford, 1969; Devey *et al*., 1981; Daniel *et al*., 1995, 1999a, b; Syrek *et al*., 1997).

A number of literature data suggest that the contribution of individual cytochrome *P*-450 (CYP) isoforms to particular metabolic pathways of phenothiazines depends on both the species and the chemical structure of phenothiazines. The metabolic studies with rat liver microsomes (Valoti *et al*., 1998) suggested that mono-N-demethylation of chlorpromazine involved CYP2D (inhibition by quinidine), while CYP1A and CYP2B participated in further demethylation of N-desmethylchlorpromazine (induction of *N*,*N*-didesmethyl-chlorpromazine formation from nor1-chlorpromazine by phenobarbital and β-naphthoflavone). Using rat CYP-specific inhibitors, Daniel *et al*. (1999a; 2002) showed that N-demethylation of different phenothiazine neuroleptics, such as promazine, thioridazine and perazine, was catalysed by the three isoforms: CYP1A2, CYP2D and CYP2B (except for CYP1A2 in the case of promazine). Formation of 5-sulphoxides of those neuroleptics depended on the drug used (promazine—CYP2D, thioridazine—CYP1A2, perazine CYP2D and CYP2B). Isoforms belonging to subfamilies CYP2C and CYP3A did not seem to be involved in the metabolism of the neuroleptics studied.

Clinical studies demonstrated that the metabolism of phenothiazine neuroleptics is under genetic control of hepatic CYP2D6 (Dahl-Puustinen *et al*., 1989; Meyer *et al*., 1990). Using human microsomes and cDNA-expressed human CYPs, Yoshii *et al*. (2000) showed that chlorpromazine 7-hydroxylation was catalysed mainly by CYP2D6, and to a lesser extent by CYP1A2. Like in the case of chlorpromazine, the aromatic hydroxylation of imipramine (a tricyclic antidepressant) was also catalysed by CYP2D6 (Brøsen *et al*., 1991; Lemoine *et al*., 1993). Studies conducted on the microsomes prepared from cells expressing recombinant human CYP2D6 and CYP3A4 suggested that thioridazine is metabolized by CYP2D6 (2- and 5-sulphoxidation), but not by CYP3A4 (Blake *et al*., 1995). Using human liver microsomes, Shin *et al*. (1999) demonstrated that phenothiazine neuroleptics (thioridazine, chlorpromazine, perphenazine) exhibited striking selectivity for CYP2D6 compared to other CYP isoforms. On the other hand, using human liver microsomes and the microsomes prepared from human ß-lymphoblastoid cell lines that had been engineered to stably express human CYP cDNA, Cashman *et al*. (1993) showed that 5-sulphoxidation of chlorpromazine was catalysed mainly by subfamily CYP3A, although CYP2A6, CYP2C8 and CYP2D6 were also likely to contribute to S-oxidase activity. In their studies performed on human liver microsomes and cDNA-expressed enzymes, Störmer *et al*. (2000) found that CYP3A4 and CYP2C9 were the major isoforms mediating perazine N-demethylation, while CYP2C19, CYP2D6 and CYP1A2 contributed to a lesser degree to that reaction.

The knowledge of contribution of particular CYP isoforms to the metabolism of promazine is of great practical value, since phenothiazine neuroleptics are administered to patients for months or years—very often in combination with antidepressants, antimanic or antianxiety drugs, to treat severe complex or 'treatment-resistant' psychiatric disorders. Such situations raise a possibility of pharmacokinetic interactions.

The aim of the present study was to investigate the contribution of human CYPs to the 5-sulphoxidation and N-demethylation of promazine, an aliphatic-type derivative and the chemically simplest phenothiazine neuroleptic. Therefore, this paper may constitute a solid basis for further enzymatic studies with phenothiazine neuroleptics with a more complex chemical structure, and may help to find a relation between the structure of phenothiazine neuroleptics and the competence of CYPs in catalysing their metabolic pathways.

Methods

Human liver samples

The use of human liver samples for scientific purposes was approved by the French National Ethics Committee. Human liver specimens were obtained from an organ donor FH61289 (livers not suitable for transplantation because of high levels of

Table 1 Clinical characteristics of patients and donors of liver specimens

Patient identification	*Age (years)*	*Gender*	*Diagnosis*
FT43	61	Female	Metastasis from colon cancer
FT80	61	Male	Metastasis from colon cancer
FT82	68	Male	Metastasis from colon cancer
FT84	43	Male	Hepatocarcinoma in normal liver
FT85	24	Female	Adenoma
FT86	58	Male	Metastasis from colon cancer
FT57			Metastasis from colon cancer
FT92	30	Male	Metastasis from colon cancer
FT95		Female	Metastasis from colon cancer
FT99	27	Male	Metastasis from colon cancer
FT100	67	Male	Metastasis from colon cancer
FT101	45	Female	Metastasis from colon cancer
FH61289	60	Male	Cerebral haemorrhage

transaminases or steatosis) and from 14 patients undergoing hepatic lobectomies (livers with normal levels of transaminases and steatosis). The clinical characteristics of the donor and patients are presented in Table 1.

Liver microsomes

Microsomes were prepared from liver samples by differential centrifugation, and were stored as described previously (Diaz *et al*., 1990). Protein concentration was determined by a bicinconinic acid method, according to the protocol provided by the manufacturer (Pierce Chemical Co., Rockford, IL, USA).

Liver microsomes from patients FH61289, FT95 and FT100 were used for optimizing the conditions of promazine metabolism. On the basis of the obtained results, promazine metabolism in liver microsomes was studied in respect of the linear dependence of product formation on time and on protein and substrate concentrations. Microsomal protein, 250 μg, was resuspended in 500 μl of 20 mM Tris/HCl buffer (pH = 7.4). To determine enzyme kinetic parameters, the promazine concentrations used ranged from 5 to 600 μM. For studies of promazine metabolism in individual patients, 25 μM promazine was used. For inhibition studies, 25 μM promazine was incubated with the specific CYP inhibitors: 10 μM furafylline (FURAF) (a CYP1A2 inhibitor), 10 μM sulfaphenazole (SULF) (a CYP2C9 inhibitor), 10 μM quinidine (QUIN) (a CYP2D6 inhibitor), 200 μM diethyldithiocarbamic acid (DDC) (a CYP2A6 + CYP2E1 inhibitor), 2 μM ketoconazole (KET) (a CYP3A4 inhibitor) and 5 μM ticlopidine (TICLOP) (a CYP2C19 inhibitor). After a 3-min preincubation at 37°C, the reaction was initiated by adding NADPH to a final concentration of 1 mM. After a 20-min incubation, the reaction was stopped by adding 200 μl of methanol. Promazine and its metabolites were analysed by a high performance liquid chromatography (HPLC) method as described below.

Correlation analysis of the data

The rates of promazine 5-sulphoxidation and N-demethylation were correlated with the rates of CYP-specific reactions (ethoxyresorufin *O*-deethylation (EROD), acetanilide 4-hydroxylation (ACT), coumarin 7-hydroxylation (COUM), *S*-mephenytoin N-demethylation (N-MEPH), tolbutamide 4-methylhydroxylation (TOLB), *S*-mephenytoin 4′-hydroxylation (OH-MEPH), chlorzoxazone 6-hydroxylation (CHLRZ), cyclosporin A oxidation (CsA) and the level of CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1, CYP3A4) obtained by a Western blot for each preparation of liver microsomes. Each pair of the data was compared by a simple linear regression analysis using the statistical programme Prism 2.01.

cDNA-expressed human CYPs

Microsomes from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Supersomes) were obtained from Gentest Corp. (Woburn, MA, USA). Promazine metabolism was studied under experimental conditions similar to those described for liver microsomes, with 10, 25, 100 and 300 μM promazine, except for the fact that the incubation time was 30 min, and the final concentration of CYPs was 100 pmol ml^{-1}. Promazine and its metabolites were analysed by an HPLC method as described below.

Primary cultures of human hepatocytes

Primary cultures of human hepatocytes were prepared as described previously (Diaz *et al*., 1990; Pichard *et al*., 1990). Ten million cells in 7 ml of a culture medium were placed in 10-cm plastic dishes precoated with collagen (Beckton-Dickinson, France). The culture medium consisted of a mixture of Ham F12 and Williams' E (1:1 v v^{-1}), supplemented as described earlier (Isom & Georgoff, 1984). The culture medium was also supplemented with 5% calf serum during the first 4 h after plating to favour the attachment of cells. Then the medium was changed and subsequently renewed every 24 h in the absence of serum. The cultures were kept at 37°C in an atmosphere of 95% air and 5% CO_2, at ca. 100% humidity. For the treatment of cells, inducers were diluted in dimethylsulphoxide and added to the culture medium at a final concentration of 25 μM for rifampicin (an inducer of CYP3A4, and to a lesser degree of CYP2B6 and CYP2C) and 10 nM for TCDD (a CYP1A1/1A2 inducer). The concentration of dimethylsulphoxide in the culture medium (of both the control and inducer-treated cultures) was 0.1%. Treatments lasted 96 h and were renewed every 24 h when the culture medium was changed.

Table 2 Kinetic parameters of promazine 5-sulphoxidation and N-demethylation in human liver microsomes (Lineweaver–Burk analysis)

Patients	*Promazine 5-sulphoxidation*		*Promazine N-demethylation*	
	K_m (μM)	V_{max} (nmol (mg protein)$^{-1}$ min^{-1})	K_m (μM)	V_{max} (nmol (mg protein)$^{-1}$ min^{-1})
FH61289	135	0.15	135	0.35
FT95	105	0.42	56	0.69
FT100	119	0.15	50	0.45
Mean$\pm$s.d.	120$\pm$15	0.24$\pm$0.15	80$\pm$47	0.50$\pm$0.17

After 96 h, the culture medium was changed for a medium without inducers (for pretreated cultures), but containing 25 μM of promazine. The cells were then incubated under standard culture conditions for 0, 2, 4, 6, 8 and 24 h. At the indicated times, 500-μl aliquots of the culture medium were collected, and 50 μl was injected directly into the HPLC system. Promazine and its metabolites were analysed by an HPLC method as described below.

Determination of promazine and its metabolites in the incubation medium

Promazine and its metabolites were quantified using the previously described HPLC method (Daniel *et al*., 1995). After incubation, the samples were centrifuged for 10 min at $2000 \times g$. The water phase containing promazine and its metabolites was extracted (pH = 12) with diethyl ether and dichloromethane (1:1, v v^{-1}). The residue obtained after evaporation of the microsomal extracts was dissolved in 100 μl of the mobile phase described below. An aliquot of 50 μl was injected into the HPLC system. The concentrations of promazine and its main metabolites (*N*-desmethylpromazine, promazine 5-sulphoxide) were assayed using a LaChrom (Merck-Hitachi) HPLC system with UV detection. The analytical column (Econosphere C18, 5 μm, 4.6 $\times$ 250 mm) was purchased from Alltech (Carnforth, England). The mobile phase consisted of an acetate buffer, pH = 3.4 (100 mmol of ammonium acetate, 20 mmol of citric acid and 1 ml of triethylamine in 1000 ml of the buffer, adjusted to pH = 3.4 with 85% phosphoric acid), and acetonitrile in the proportion 50:50. The flow rate was 1.2 ml min^{-1}, the column temperature was 40°C. The absorbance of promazine and its metabolites was measured at a wavelength of 260 nm. The compounds were eluted in the following order: promazine 5-sulphoxide (4.77 min), *N*-desmethylpromazine (7.80 min), promazine (10.01 min).

Drugs and chemicals

Promazine hydrochloride was obtained from Polfa, Jelenia Góra, Poland. Desmethylpromazine was donated by Professor M.H. Bickel, the University of Bern, Switzerland. Promazine sulphoxide was synthesized according to the previously described method (Daniel *et al*., 1995), based on that by Ovens *et al*. (1989). QUIN, SULF, TICLOP, KET, DDC and rifampicin were purchased from Sigma, St Louis, U.S.A. FURAF was provided by Ultrafine Chemicals, Manchester, U.K. 2,3,7,8-tetrachlorodibenzeno-*p*-dioxin (TCDD) was obtained from ChemSyn Science Laboratories, Lenaxa, U.S.A. NADPH was from Boehringer Mannheim GmbH, Germany. Bovine serum albumin was obtained from Pierce Chemical Co., Rockford, U.S.A. All the organic solvents with HPLC purity were supplied by Carlo Erba, Milan, Italy.

Results

Kinetic parameters of promazine metabolism in human liver microsomes

The Lineweaver – Burk plots were linear ($r^2 = 0.972 - 0.998$), which allowed graphic estimation of the values for Michaelis constant (K_m) and maximum velocity of the reaction (V_{max}) for promazine 5-sulphoxidation and N-demethylation in three different human liver microsomes (Table 2).

However, Figures 2a and b show the representative Eadie – Hofstee plots for promazine 5-sulphoxidation and N-demethylation in liver microsomes of patient FT95. These plots suggest that multiple enzymes are responsible for the biotransformation of promazine via 5-sulphoxidation and N-demethylation. Similar results were obtained with microsomes of patients FT99 and FT100. Therefore, kinetic parameters shown in Table 2 are resultants of K_m and V_{max} values of individual enzymes contributing to the processes studied.

Correlation study

A total of 15 different preparations of human liver microsomes were used to evaluate the interindividual variability of promazine biotransformation. The microsome preparations produced different amounts of promazine 5-sulphoxide and *N*-desmethylpromazine (Figure 3). As a rule, promazine 5-sulphoxide was produced in smaller amounts compared to *N*-desmethylpromazine, which is in agreement with earlier investigations on rats (Goldenberg *et al*., 1965; Daniel *et al*., 1995, 1999a, b; Syrek *et al*., 1997). Interindividual differences in the rates of promazine metabolism were up to three-fold as high, which agrees with the kinetics parameters in Table 2. The average velocities were 43.2$\pm$13 pmol (mg protein)$^{-1}$ min^{-1} in the case of promazine 5-sulphoxide and 102.2$\pm$41.8 pmol (mg protein)$^{-1}$ min^{-1} in the case of *N*-desmethylpromazine.

The microsomes from the bank, used in this study, were tested for the levels of several CYPs determined by immunoblotting, and for several monooxygenase activities (data not shown). The rate of formation of promazine metabolites was compared with CYP levels and monooxygenase activities. The results of such analyses are shown in Table 3, where the correlation coefficient (R) and the P-values are quoted for each pair of data. The formation of promazine 5-sulphoxide correlated significantly with the level of CYP1A2 ($P = 0.013$) and with ethoxyresorufin *O*-deethylase activity ($P = 0.0024$)

and acetanilide 4-hydroxylase activity ($P=0.042$), as well as with the level of CYP3A4 ($P=0.0005$) and with cyclosporin A oxidase activity ($P=0.0096$). The production of *N*-desmethylpromazine correlated highly with the level of CYP1A2 ($P=0.0058$) and with ethoxyresorufin *O*-deethylase activity ($P<0.0001$), but showed a weaker correlation with acetanilide 4-hydroxylase activity ($P=0.029$). The formation of *N*-desmethylpromazine also correlated significantly with *S*-mephenytoin 4′-hydroxylase activity ($P=0.030$), as well as with the level of CYP3A4 ($P=0.020$) and cyclosporin A oxidase activity ($P=0.046$). No correlation was observed between the production of promazine metabolites and the levels of CYP2A6, CYP2B6, CYP2C9, CYP2D6, or the rates of COUM, N-MEPH, CHLRZ and TOLB.

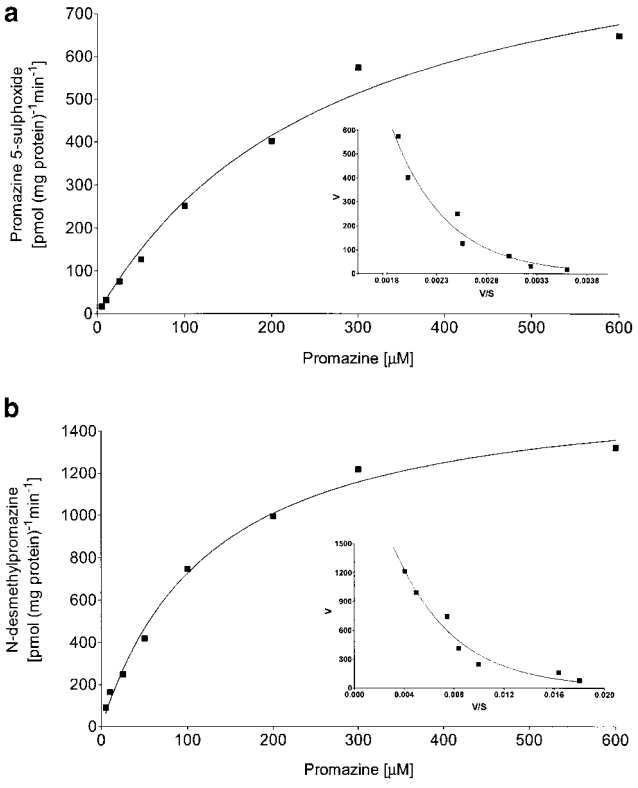

Figure 2 Formation of promazine 5-sulphoxide (a) and *N*-desmethylpromazine (b) from promazine. Human liver microsomes of patient FT95 (0.5 mg of protein ml^{-1}) were incubated in a 20 mM Tris/HCl buffer (pH = 7.4) with promazine (5 – 600 μM) and NADPH (1 mM) for 20 min. The insets show Eadie – Hofstee plots.

Inhibition of promazine metabolism by specific CYP inhibitors

For inhibition studies, microsomes from patients FT95 and FT99 were used. In the case of the preparation of FT95, FURAF (a CYP1A2 inhibitor) and KET (a CYP3A4 inhibitor) inhibited promazine 5-sulphoxidation up to 72 and 65% of the control activity, respectively (Figure 4a). At the same time, FURAF exerted a strong inhibitory effect on the rate of promazine N-demethylation (up to 55% of the control activity), while TICLOP inhibited that reaction up to 75% of the control value (Figure 4a). Similar effects of specific CYP inhibitors on the rates of promazine metabolism were observed in the case of preparation FT99 (Figure 4b). DDC, SULF and QUIN exhibited no inhibitory effect or only marginally inhibited the two metabolic pathways of promazine (Figures 4a and b).

Study with cDNA-expressed human CYPs

The ability of cDNA-expressed human CYPs to metabolize promazine at its low concentration (10 μM) is shown in Figures 5a and b. The preference of CYP isoforms for catalyzing promazine metabolism was as follows: 1A1 > 2B6 > 1A2 > 2C9 > 3A4 > 2E1 > 2A6 > 2D6 > 2C19 for 5-sulphoxidation, and 2C19 > 2B6 > 1A1 > 1A2 > 2D6 > 3A4 > 2C9 > 2E1 > 2A6 for

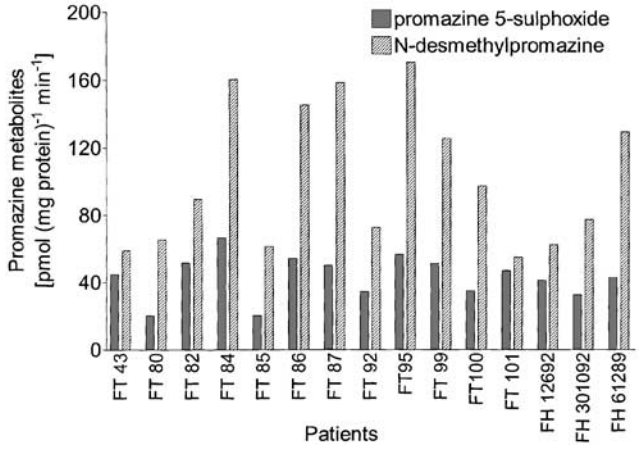

Figure 3 Interindividual variability of promazine metabolism in human liver microsomes. Human liver microsomes (0.5 mg of protein ml^{-1}) were incubated in a 20 mM Tris/HCl buffer (pH = 7.4) with promazine (25 μM) and NADPH (1 mM) for 20 min.

Table 3 A correlation (*R* and *P*-values) between the rate of promazine 5-sulphoxidation and N-demethylation and the velocity of CYP-specific reactions or the level of CYPs in human liver microsomes

		EROD	*ACT*	*1A2*	*COUM*	*2A6*	*N-MEPH*	*2B6*	*TOLB*	*2C9*	*OH-MEPH*	*2D6*	*CHLRZ*	*2E1*	*CsA*	*3A4*
Promazine	R	0.721	0.529	0.623	0.380	0.462	0.279	0.434	−0.364	−0.359	−0.013	0.185	0.344	0.400	0.644	0.786
5-sulphoxide	P	*0.0024*	*0.042*	*0.013*	0.162	0.083	0.321	0.107	0.182	0.189	0.964	0.508	0.209	0.139	*0.0096*	*0.0005*
N-desmethyl	R	0.849	0.564	0.674	0.252	−0.075	0.275	0.253	−0.169	−0.089	0.559	−0.099	0.362	0.182	0.521	0.593
promazine	P	*<0.0001*	*0.029*	*0.0058*	0.365	0.790	0.328	0.371	0.547	0.751	*0.030*	0.726	0.184	0.516	*0.046*	*0.020*

EROD, ethoxyresorufin *O*-deethylation; ACT, acetanilide 4-hydroxylation; COUM, coumarin 7-hydroxylation; N-MEPH, *S*-mephenytoin *N*-demethylation; TOLB, tolbutamide 4-methylhydroxylation; OH-MEPH, - *S*-mephenytoin 4′-hydroxylation; CHLRZ, chlorzoxazone 6-hydroxylation; CsA, cyclosporin A oxidation. Each pair of data was compared by a simple linear regression analysis using the statistical programme Prism 2.01.

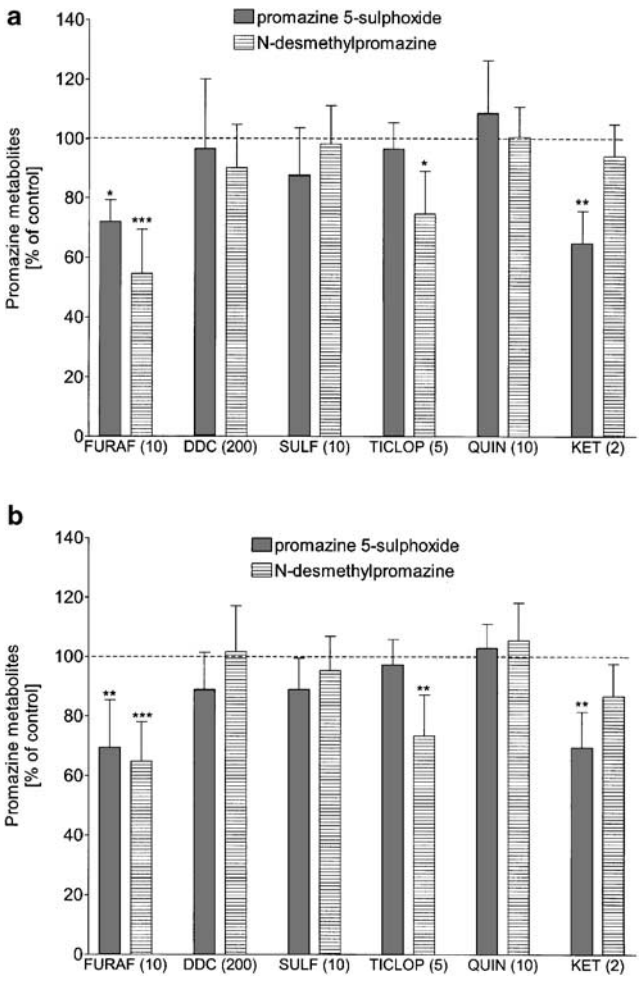

Figure 4 Effect of CYP-specific inhibitors on the rate of promazine 5-sulphoxidation and N-demethylation in human liver microsomes of patient FT95 (a) and FT99 (b). Human liver microsomes were incubated with 25 μM promazine, in the absence (control) or presence of CYP-specific inhibitors: 10 μM furafylline (FURAF), 200 μM diethyldithiocarbamic acid (DDC), 10 μM sulfaphenazole (SULF), 5 μM ticlopidine (TICLOP), 10 μM quinidine (QUIN) and 2 μM ketoconazole (KET). Absolute control values were 57 ± 13 pmol of promazine 5-sulphoxide (mg protein)$^{-1}$min^{-1} and 174 ± 18 pmol of *N*-desmethylpromazine (mg protein)$^{-1}$ min^{-1} (a), and 36 ± 6 pmol of promazine 5-sulphoxide (mg protein)$^{-1}$ min^{-1} and 128 ± 9 pmol of *N*-desmethylpromazine (mg protein)$^{-1}$ min^{-1} (b). Mean values $\pm$ s.d. ($n = 5$) are presented. Statistical significance was assessed using Student's *t*-test and indicated with $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. For further explanation see Figure 3.

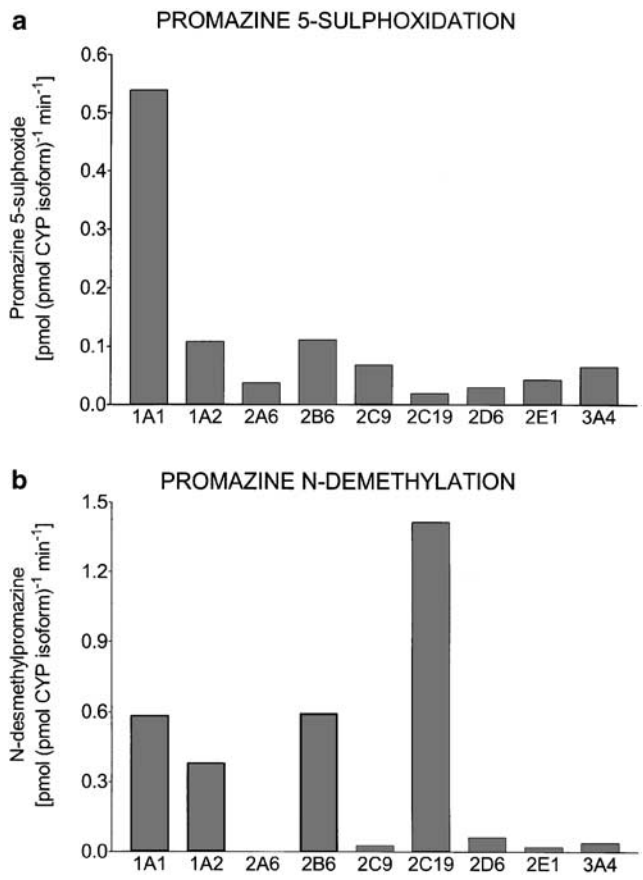

Figure 5 Biotransformation of promazine via 5-sulphoxidation (a) and N-demethylation (b) by the cDNA-expressed human CYPs (Supersomes). Promazine (10 μM) was incubated with Supersomes (100 pmol of CYP ml^{-1}) and NADPH (1 mM) for 30 min.

N-demethylation. *N*-desmethylpromazine was generated in ca. 2 – 8-fold greater amount compared to promazine 5-sulphoxide by most of the CYPs studied. In contrast, CYP2C9, CYP2E1 and CYP3A4 formed promazine 5-sulphoxide in amounts ca. two-fold greater than *N*-desmethylpromazine. CYP2A6 did not produce *N*-desmethylpromazine in a measurable amount.

The Lineweaver – Burk analysis of the promazine 5-sulphoxidation and N-demethylation in cDNA-expressed human CYPs is presented in Table 4. The obtained kinetic parameters showed distinct inter-isoform differences, which was consistent with the multienzyme Eadie – Hofstee plots derived from liver microsomes (Figure 2a and b). The highest intrinsic clearance (V_{max}/K_m) was found for CYP1A subfamily, CYP3A4 and CYP2B6 in the case of 5-sulphoxidation, and for CYP2C19, CYP1A subfamily and CYP2B6 in the case of N-demethylation (Table 4).

Biotransformation of promazine in primary cultures of human hepatocytes: effect of CYP inducers

Treatment of primary cultures of human hepatocytes with TCDD (a CYP1A subfamily inducer) and rifampicin (a CYP3A4, CYP2B6 and CYP2C inducer) increased the rate of promazine biotransformation. Approximately 80% of promazine was metabolized during the first 8 h in inducer-treated cultures (Figure 6a). Promazine 5-sulphoxide reached higher concentrations in the extracellular medium compared to *N*-desmethylpromazine (Figures 6b and c). TCDD and rifampicin induced the formation of promazine 5-sulphoxide and *N*-desmethylpromazine. The accumulation of promazine 5-sulphoxide in the extracellular medium was increased up to 6 h by TCDD or up to 8 h by rifampicin. In the case of *N*-desmethylpromazine, its concentration in the extracellular medium was increased up to 4 h by TCDD, or up to 6 h by

Table 4 Kinetic parameters of promazine 5-sulphoxidation and N-demethylation in cDNA-expressed human CYPs (Supersomes)

CYPs	*Promazine 5-sulphoxidation*			*Promazine N-demethylation*		
	K_m (μM)	V_{max} (*pmol (pmol CYP isoform)$^{-1}$ min^{-1}*)	V_{max}/K_m	K_m (μM)	V_{max} (*pmol (pmol CYP isoform)$^{-1}$ min^{-1}*)	V_{max}/K_m
CYP1A1	37	2.47	0.0667	47	3.51	0.0747
CYP1A2	46	0.61	0.0133	55	2.51	0.0456
CYP2A6	58	0.26	0.0045	741	0.65	0.0009
CYP2B6	83	0.58	0.0070	293	10.75	0.0367
CYP2C9	61	0.31	0.0051	157	0.41	0.0026
CYP2C19	71	0.15	0.0021	85	13.70	0.161
CYP2D6	350	0.74	0.0021	400	2.75	0.0007
CYP2E1	64	0.27	0.0042	159	0.33	0.0021
CYP3A4	50	0.42	0.0084	175	0.75	0.0043

Table 5 Estimation of the contribution of CYP isoforms to the 5-sulphoxidation of promazine in liver microsomes on the basis of the rates of this reaction in Supersomes

CYPs	*Relative contribution of the isoform to the total CYP contents in liver microsomes (fraction)*[a]	*Velocity in Supersomes (pmol promazine 5-sulphoxide (pmol CYP isoform)$^{-1}$ min^{-1})*		*Predicted velocity in liver microsomes (pmol promazine 5-sulphoxide (pmol of total CYP)$^{-1}$ min^{-1})*[b]		*Relative contribution of the isoform to promazine 5-sulphoxidation in liver microsomes (%)*[c]	
		Promazine		*Promazine*		*Promazine*	
		10 μM	*300 μM*	*10 μM*	*300 μM*	*10 μM*	*300 μM*
CYP1A1 (not constitutive)	—	0.539	2.555	—	—	—	—
CYP1A2	0.127	0.108	0.592	0.0137	0.0752	30.995	31.372
CYP2A6	0.040	0.037	0.211	0.0015	0.0084	3.394	3.504
CYP2B6	0.002	0.111	0.893	0.0002	0.0018	0.452	0.751
CYP2C9	0.156	0.068	0.212	0.0064	0.0331	14.48	13.808
CYP2C19	0.026	0.019	0.188	0.0005	0.0049	1.131	2.044
CYP2D6	0.015	0.029	0.363	0.0004	0.0054	0.905	2.253
CYP2E1	0.066	0.043	0.272	0.0028	0.0179	6.335	7.468
CYP3A4	0.288	0.065	0.323	0.0187	0.0930	42.308	38.798

[a]Data according to Shimada *et al*. (1994) and Maurel (1998). [b]The predicted velocity in liver microsomes was calculated by multiplying the velocity in Supersomes by the relative contribution of isoform to the total CYP content in liver microsomes. [c]Relative contribution of CYPs to promazine 5-sulphoxidation was calculated as a percentage of the sum of predicted velocities in liver microsomes.

Table 6 Estimation of the contribution of CYP isoforms to the N-demethylation of promazine in liver microsomes on the basis of the rates of this reaction in Supersomes

CYPs	*Relative contribution of the isoform to the total CYP contents in liver microsomes (fraction)*[a]	*Velocity in Supersomes (pmol N-desmethylpromazine (pmol CYP isoform)$^{-1}$ min^{-1})*		*Predicted velocity in liver microsomes (pmol N-desmethyl-promazine (pmol of total CYP)$^{-1}$ min^{-1})*[b]		*Relative contribution of the isoform to promazine N-demethylation in liver microsomes (%)*[c]	
		Promazine		*Promazine*		*Promazine*	
		10 μM	*300 μM*	*10 μM*	*300 μM*	*10 μM*	*300 μM*
CYP1A1 (not constitutive)	—	0.584	3.403	—	—	—	—
CYP1A2	0.127	0.38	2.127	0.0483	0.2701	46.622	35.252
CYP2A6	0.040	n.d.	0.184	—	0.0074	—	0.966
CYP2B6	0.002	0.593	7.187	0.0012	0.0144	1.158	1.879
CYP2C9	0.156	0.026	0.411	0.0040	0.0641	3.861	8.366
CYP2C19	0.026	1.413	9.523	0.0367	0.2476	35.425	32.315
CYP2D6	0.015	0.061	2.907	0.0009	0.0436	0.869	5.69
CYP2E1	0.066	0.020	0.336	0.0013	0.0222	1.255	2.897
CYP3A4	0.288	0.039	0.389	0.0112	0.0968	10.811	12.634

[a,b]See Table 5. [c]Relative contribution of CYPs to promazine N-demethylation was calculated as a percentage of the sum of predicted velocities in liver microsomes. n.d., not detected.

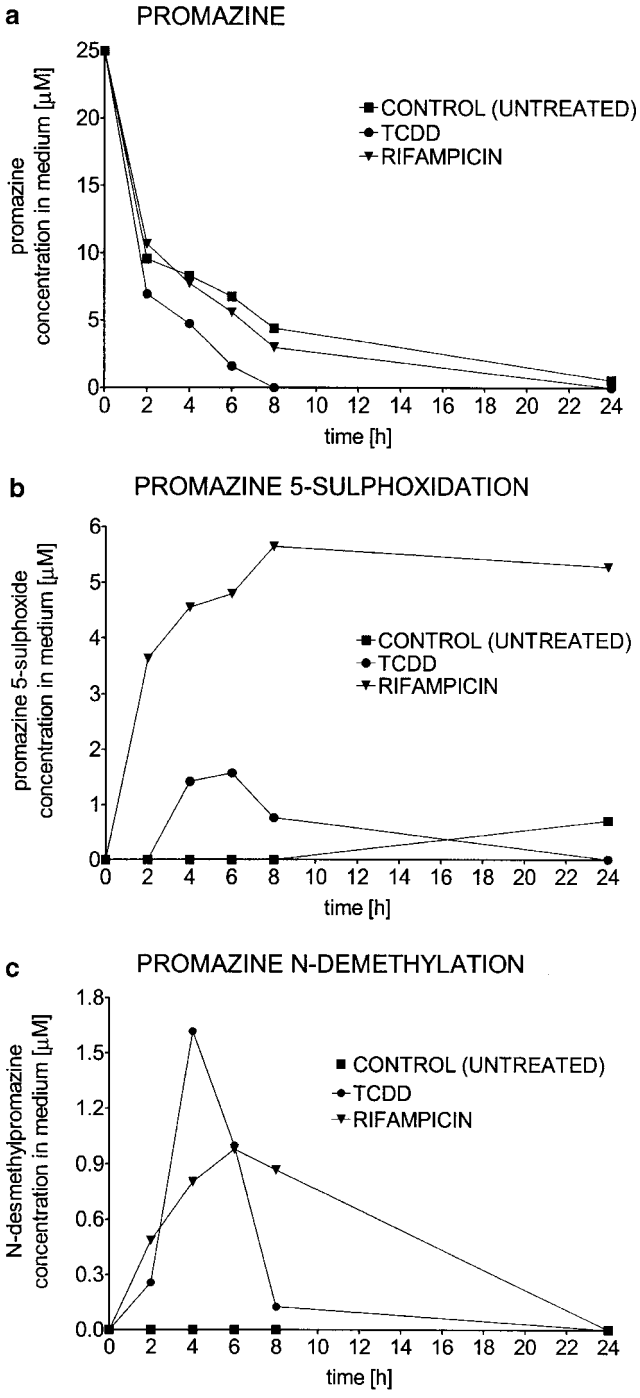

Figure 6 Influence of TCDD (a CYP1A1/A2 inducer) and rifampicin (a CYP3A4 inducer) on promazine metabolism in a primary culture of human hepatocytes. Human hepatocytes were kept in the primary culture in the absence (control) or presence of 10 nM TCDD or 25 μM rifampicin for 96 h. The medium was then renewed in the absence of the inducer, but in the presence of 25 μM promazine. The concentrations of promazine (a), promazine 5-sulphoxide (b) and *N*-desmethylpromazine (c) formed from neuroleptic were measured in the culture medium.

rifampicin. After 24 h, promazine and its metabolites were not detected in the extracellular medium of the inducer-treated cultures, except for promazine 5-sulphoxide in the rifampicin-treated cultures (the detection threshold for promazine or its metabolites was as low as 0.01 nmol per sample).

Discussion

The results presented above indicate major contribution of CYP1A2 and CYP3A4 to promazine 5-sulphoxidation, and of CYP1A2 and CYP2C19 to its N-demethylation. CYP2C9 and CYP3A4 contribute to a lesser degree to the 5-sulphoxidation and N-demethylation, respectively, of the neuroleptic. This conclusion is based on the following arguments. First, in the bank of human liver microsomes, the formation of promazine 5-sulphoxide and *N*-desmethylpromazine was significantly correlated with: (a) the level of CYP3A4, and cyclosporin A oxidase activity; (b) the level of CYP1A2, and the activities of ethoxyresorufin *O*-deethylase and acetanilide 4-hydroxylase. Moreover, promazine N-demethylation correlated positively with the activity of *S*-mephenytoin 4′-hydroxylase (CYP2C19). Second, the production of promazine 5-sulphoxide was significantly reduced by FURAF (a CYP1A2 inhibitor) and KET (a CYP3A4 inhibitor), while the production of N-desmethylpromazine was significantly decreased by FURAF (a CYP1A2 inhibitor) and TICLOP (a CYP2C19 inhibitor). Third, of the cDNA-expressed human CYPs studied, the highest intrinsic clearances (V_{max}/K_m) and rates of promazine metabolism were found for the CYP1A subfamily and CYP2B6 (for both the reactions), as well as for CYP2C19 (for N-demethylation) and CYP3A4 (for 5-sulphoxidation); however, considering the relative amount of each isoform in the total CYP content in the liver, the role of CYP1A2, CYP3A4 and CYP2C19 in promazine metabolism seems to be predominant (Tables 5 and 6). Fourth, in the primary cultures of human hepatocytes, rifampicin (a CYP3A4 inducer) and TCDD (a CYP1A subfamily inducer) induced the formation of both those promazine metabolites, rifampicin being a more potent inducer of 5-sulphoxidation than of N-demethylation. The latter observation suggests that CYP3A4 is more important for promazine 5-sulphoxidation than for its N-demethylation.

Of the microsomes used in correlation studies, some were deficient in CYP2D6 (FT80, FH12692); however, those preparations were as active as the others in the formation of promazine metabolites. Accordingly, QUIN (a CYP2D6 inhibitor) did not affect the biotransformation of promazine in extensive metabolizers. On the other hand, the Supersomes CYP2D6 were able to generate both the promazine metabolites. However, in humans CYP2D6 contributes less than 5% to the total amount of CYP in the liver (Shimada *et al*., 1994; Maurel, 1998). Hence the relative contribution of CYP2D6 to the 5-sulphoxidation and N-demethylation of promazine *in vivo* is very low. Our results are consistent with those of Störmer *et al*. (2000) who reported that in human liver microsomes CYP2D6 contributed to the N-demethylation of perazine to a lesser extent than did CYP3A4. Other studies demonstrated that CYP2D6 was involved mainly in the aromatic hydroxylation of chlorpromazine and imipramine (Brøsen *et al*., 1991; Lemoine *et al*., 1993; Yoshi *et al*., 2000).

Thus, our results obtained with cDNA-expressed human CYPs showed that all the isoforms tested generated detectable, but different, amounts of promazine 5-sulphoxide and N-

desmethylpromazine, which suggests a nonspecific catalysis of promazine metabolism. However, in liver microsomes or *in vivo* the amount of a metabolite formed by an individual isoform depends on both the catalytic activity and the contribution of an isoform to the total CYP content in the liver. It has been reported that the relative contribution of CYP1A2 and CYP3A4 to the total CYP content in human liver microsomes amounts to 13 and 29%, respectively, while CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 constitute 4, 0.2, 16, 2.5, 1.5 and 7% of the total CYP protein, respectively (Shimada *et al*., 1994; Maurel, 1998). Accordingly, the contribution of CYP3A4 and CYP1A2 to the 5-sulphoxidation and N-demethylation of promazine in the whole liver should be relatively greater than in the Supersomes. Therefore, we attempted to estimate roughly the contribution of the CYP isoforms studied to the 5-sulphoxidation and N-demethylation of promazine in liver microsomes on the basis of the rate of those reactions in the Supersomes (Tables 5 and 6). Our calculations performed at low (10 μM) and at high (300 μM) concentration of promazine indicate that CYP1A2 and CYP3A4 are the main isoforms responsible for 5-sulphoxidation, while CYP1A2 and CYP2C19 are the main isoforms that catalyse promazine N-demethylation in the liver. Moreover, of the other isoforms studied, CYP3A4 and CYP2C9 contribute to a lesser degree to promazine N-demethylation and 5-sulphoxidation, respectively. The role of other CYP isoforms in the investigated metabolic pathways of promazine seems negligible; however, it may increase at a higher, nontherapeutic concentration of the drug (Tables 5 and 6). The calculated data agree with other authors' results showing that the CYP3A subfamily is mainly involved in the 5-sulphoxidation of chlorpromazine (Cashman *et al*., 1993). The calculated data (Tables 5 and 6) are not consistent with the findings of Störmer *et al*. (2000) indicating that CYP3A4 and CYP2C9 are the main isoforms catalyzing the N-demethylation of perazine, while CYP1A2 and CYP2C19 play a minor role in this process. The observed discrepancy may be because of different structures of the phenothiazines studied, which influence their access to and interaction with the catalytic sites of CYPs. Using rat CYP-specific inhibitors, Daniel *et al*. (1999a; 2002) showed that the 5-sulphoxidation of promazine, perazine and thioridazine in rats is catalysed by different isoforms: CYP2D, CYP2D + CYP2B and CYP1A2, respectively. The observed discrepancies between species in the participation of CYPs in the catalysis of metabolism of phenothiazine neuroleptics may be partly due to interspecies differences in the relative contribution of individual isoforms to the total amount of CYP (Shimojo *et al*., 1993; Shimada *et al*., 1994), and in the specificity of catalytic sites of CYP1A2 and CYP2D in the liver (Steiner *et al*., 1988; Zhi-Guang *et al*., 1988; Kobayashi *et al*., 1989; Boobis *et al*., 1990; Sesardic *et al*., 1990).

In summary, CYP1A2 and CYP3A4 are the main isoforms responsible for 5-sulphoxidation, while CYP1A2 and CYP2C19 mainly catalyse the N-demethylation of promazine in human liver. Moreover, CYP2C9 and CYP3A4 also contribute to promazine 5-sulphoxidation and N-demethylation, respectively. Thus, the catalysis of promazine N-demethylation is similar to that of imipramine, a dibenzoazepine analogue of promazine (Skjelbo *et al*., 1991; Lemoine *et al*., 1993). In both these cases CYP1A2, CYP2C19 and CYP3A4 are the main enzymes that catalyse the process of N-demethylation. These findings may have significant implications for the prediction of potential drug – drug interactions involving promazine. Phenothiazine neuroleptics are combined with antidepressants or carbamazepine in the treatment of complex or 'treatment-resistant' psychiatric disorders. Thus promazine may compete with tricyclic antidepressants for the active centres of CYP1A2, CYP3A4 and CYP2C19. Moreover, its metabolism via CYP1A2 may be inhibited by fluvoxamine, and that via CYP2C19 and CYP3A4 by fluvoxamine and fluoxetine (Brøsen *et al*., 1993; Kobayashi *et al*., 1995; Jeppesen *et al*. 1996). On the other hand, metabolism of promazine mediated by CYP3A4 may be induced by carbamazepine. Interactions of this type between promazine and antidepressant drugs or carbamazepine have been observed in the rat (Syrek *et al*., 1996, 1997; Daniel *et al*., 1999b). It is also important to note that the metabolism of promazine may be dependent on the known CYP2C19 polymorphism occuring at the highest rate in Oriental populations.

This study was supported by the Poste Jaune fellowship granted to Jacek Wójcikowski by the INSERM, Paris, and by the INSERM U128, Montpellier, France; support also came from the statuatory funds of the Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland.

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(Received December 20, 2002
Accepted January 16, 2003)