Identification of the human cytochrome P450 isoforms mediating *in vitro* N-dealkylation of perphenazine

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Aims To identify the human cytochrome P450 (CYP) isoforms mediating the N-dealkylation of the antipsychotic drug perphenazine *in vitro* and estimate the relative contributions of the CYP isoforms involved.

Methods cDNA-expressed CYP isoforms were used to identify the isoforms that are able to mediate the N-dealkylation of perphenazine, which is considered a major metabolic pathway for the drug. Using human liver microsomal preparations (HLM), inhibition studies were carried out to establish the relative contributions of the CYP isoforms involved in the N-dealkylation reaction.

Results CYP isoforms 1A2, 3A4, 2C8, 2C9, 2C18, 2C19 and 2D6 were able to mediate the N-dealkylation of perphenazine. Reaction velocities and their relative abundance in HLM suggested that CYP1A2, 3A4, 2C19 and 2D6 were the most important contributors to N-dealkylation. Apparent K_m values of CYP1A2 and CYP2D6 were in the range 1–2 μM , and K_m values of CYP2C19 and CYP3A4 were 14 μM and 7.9 μM , respectively. Ketoconazole inhibition of N-dealkylation mediated by a mixed HLM indicated that CYP3A4 accounted for about 40% of perphenazine N-dealkylation at therapeutically relevant concentrations. The contribution of the CYP isoforms 1A2, 2C19 and 2D6 amounted to 20–25% each as measured by the percentage inhibition obtained by addition of furafylline, fluvoxamine or quinidine, respectively. HLM-mediated N-dealkylation of perphenazine accounted for 57% of the total amount of substrate consumed during incubation.

Conclusions The present *in vitro* study suggests that CYP isoforms 1A2, 3A4, 2C19 and 2CD6 are primarily involved in the N-dealkylation of perphenazine. The relatively modest role of CYP2D6 is at variance with *in vivo* studies, which indicate a greater contribution of this isoform. Alternative metabolic pathways, corresponding to 43% of the HLM-mediated metabolism of the drug, may depend more strongly on CYP2D6.

Keywords: cytochrome P450, human liver microsomes, N-dealkylation, perphenazine

Introduction

Perphenazine is a phenothiazine derivative, which was developed in the years following the introduction of chlorpromazine as an antipsychotic drug in 1952. The metabolism and urinary excretion of perphenazine have been studied both in rats and man, and measurements of the concentration of perphenazine in serum are used for

therapeutic drug monitoring (TDM) [1–7]. The major steps in the metabolism of perphenazine in humans are N-dealkylation leading to loss of the hydroxyethyl group, oxidation of the sulphur atom and ring hydroxylation at position 7 (Figure 1). Minor metabolic pathways may be N-oxidation and direct glucuronidation of the primary alcohol group [3, 8]. Receptor binding studies indicate that 7-OH-perphenazine has about 70% of the antipsychotic activity of the parent compound, while the other metabolites are considered to be inactive [9]. Determination of perphenazine and metabolites in serum from patients at steady-state have shown concentrations of N-dealkylated perphenazine corresponding to three times those of perphenazine, whereas perphenazine

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Figure 1 The major routes of perphenazine metabolism.

sulphoxide concentrations were in the same range as those of the parent compound [10]. No information about the concentration of 7-OH-perphenazine in serum is available, but this compound is excreted in the urine mainly as the glucuronide [3, 8]. *N*-dealkylated perphenazine is eliminated as its sulphoxide, whereas about 13% of a dose is excreted in the urine as perphenazine-sulphoxide [4].

The cytochrome P450 (CYP) isoforms involved in the metabolism of perphenazine have not been studied systematically, but indirect evidence points towards a role for CYP2D6. Perphenazine acts as an inhibitor of the conversion of nortriptyline to (*E*)-10-hydroxy-nortriptyline, a process almost exclusively mediated by CYP2D6 at therapeutic nortriptyline concentrations, and this may lead to a clinically significant increase of the plasma nortriptyline concentration in patients treated with both drugs [11–13]. Serum perphenazine concentration is increased by addition of the selective serotonin reuptake inhibitor (SSRI) paroxetine, which is known as a potent inhibitor of

CYP2D6 [14]. Finally, higher serum levels of perphenazine corrected for dose have been found in patients lacking CYP2D6 (poor metabolizers) than in patients having a functional enzyme (extensive metabolizers) [15, 16]. Other CYP isoforms, however, are also likely to contribute to the metabolism of perphenazine. Thus, a considerable interindividual variation of the concentration to dose ratio (C/D) at steady-state has been found in both CYP2D6 extensive and poor metabolizers leading to a considerable overlap of this ratio in the two groups [16]. This pattern together with the finding that the median C/D was only increased by 100% in poor metabolizers points towards the involvement of additional enzymes.

In the present *in vitro* study the human CYP isoforms mediating the *N*-dealkylation of perphenazine were identified. In human liver microsomal preparations (HLM), the relative individual contributions of the CYP isoforms involved in the *N*-dealkylation of perphenazine were determined by defining the effect of selective inhibitors on the formation of *N*-dealkylated perphenazine. The relative importance of *N*-dealkylation to the total metabolism of perphenazine by HLM was also assessed.

Methods

Chemicals and reagents

N-dealkylated perphenazine was a gift from Schering-Plough Research Institute (Kenilworth, NJ). Fluvoxamine, ketoconazole and (*E*)-10-hydroxy-nortriptyline (used as internal standard) were donated by Solvay Duphar B.V. (Weesp, the Netherlands), Janssen Pharmaceutica N.V. (Beerse, Belgium) and Lundbeck A/S (Copenhagen, Denmark), respectively. Quinidine and perphenazine were from Sigma Chemical Co. (St Louis, MO), and furafylline was obtained from Ultrafine Chemicals (Manchester, UK). Other chemicals were of analytical grade. cDNA-expressed human CYPs 1A2, 3A4, 2C8, 2C9, 2C18, 2C19, 2E1 and 2D6, prepared from baculovirus infected insect cells, were purchased from Gentest Co. (Woburn, MA). Human liver microsomal preparations (HLMs) were also obtained from Gentest Co. According to the supplier, all donors were Caucasians, and the specimens were obtained *post mortem*. One HLM (H161Pool, Lot 6) was a pool prepared as a mixture of microsomes from 10 different donors. Gender was equally represented in this pool. Three other HLMs (HG23, HG30 and HG112) were from individual donors. The concentrations of the individual CYP isoforms in the HLMs have been determined by Western blotting by the supplier (Table 1). With regard to the CYP2C family, only the content of CYP2C9 was determined. The

Table 1 The CYP isoform content (pmol mg⁻¹protein) of the pooled and the individual human liver microsomal preparations as determined by Western blotting using cDNA-expressed enzymes as standards (according to Gentest Co).

	H161Pool	HG23	HG30	HG112
CYP1A2	50	50	117	3
CYP3A ¹	127	38	129	331
CYP2C9	55	40	106	72
CYP2E1	53	52	27	70
CYP2D6	11	12	n.d. ²	8

¹Principally CYP3A4. ²Not detectable

microsomal preparations were received frozen and were stored at -80°C until use.

Experimental procedure

The enzymatic reactions were carried out at pH 7.4 in propylene tubes at 37°C in a total volume of 300 µl of 100 mM phosphate buffer as described previously [17]. The NADPH generating system consisted of 1 U isocitrate dehydrogenase, 5 mM NADP, 5 mM isocitrate and 5 mM MgCl₂. The reaction was started by addition of 50–200 µg HLM protein or 2.5–10 pmol of the individual CYP isoforms and stopped 10 min later by addition of 1 ml ice-cold 1 M carbonate/bicarbonate buffer, pH 10.5. Control values were obtained at all substrate concentrations by adding the stop reagent before the microsomes. In order to assess the CYP isoforms involved in the N-dealkylation of perphenazine and to study the linearity of N-dealkylated perphenazine formation, incubation times up to 60 min were used for substrate concentrations of 2, 10 and 100 µM. For all processes proportionality was found between the amount of enzyme added and the product formed, and N-dealkylated perphenazine formation was linear up to about 12 min of incubation.

Inhibitor studies

Chemical inhibitors were added to the reaction medium in order to determine the quantitative contribution of the individual CYP isoforms to the formation of total N-dealkylated perphenazine in HLM. Furafylline, ketocozazole, fluvoxamine and quinidine were used as inhibitors of CYP1A2, 3A4, 2C19 and 2D6, respectively [18–23]. Furafylline was used as a competitive inhibitor in the present study (23). This inhibitor is almost insoluble in water but soluble in dimethyl sulphoxide (DMSO). A final concentration of 1% DMSO was used in both the control (uninhibited) reaction medium and the medium containing the inhibitor. The specificity of CYP inhibitors is usually only relative and depends on the concentration of

an inhibitor. The activity of several CYP isoforms may be inhibited to various extents by an individual inhibitor [22]. Furthermore, given a competitive mechanism of inhibition, the extent of inhibition of the reaction rate depends on the substrate concentration, K_m , concentration of the inhibitor and the affinity of the inhibitor K_i [24]. Thus, initial inhibition experiments were carried out to determine suitable inhibitor concentrations in the present context.

Assay of perphenazine and dealkylated perphenazine

The N-dealkylated perphenazine formed during the incubation period was determined by h.p.l.c. essentially as described previously for the determination of citalopram metabolites [25]. After addition of 50 µl 3 µM (*E*)-10-OH-nortriptyline as internal standard, N-dealkylated perphenazine in the incubate was extracted into 7.5 ml heptane-isoamylalcohol (97.5 : 1.5; v/v) by shaking. After centrifugation the aqueous phase was frozen by immersing the tubes in a mixture of dry ice and ethanol. The organic phase was transferred to new tubes, and the analytes were back-extracted into 125 µl of 250 mM phosphoric acid, of which 75 µl was injected onto a h.p.l.c. instrument equipped with a Hypersil BDS C₁₈ column. The mobile phase was an acetonitrile-44 mM KH₂PO₄ buffer, pH 2.5 (67.5 : 32.5; v/v), which contained 10 mM triethylamine. The lower level of quantification was 3 pmol N-dealkylated perphenazine. The interday precision was <8%.

Analysis of data

Reaction velocities (*V*) were expressed as nmol h⁻¹ mg⁻¹ protein, when HLM was used, and as nmol h⁻¹ nmol⁻¹ CYP, when the activities of the CYP isoforms were measured. Apparent K_m and V_{max} values were calculated by nonlinear regression analysis according to the Michaelis-Menten equation using GraphPad Prism software (San Diego, CA). An *F*-test indicated whether a one- or two-binding site model was optimal. An Eadie-Hofstee plot, *V* vs *V*/*S*, where *S* is the substrate concentration, was used to illustrate multiple binding sites [26]. Degrees of inhibition were expressed as a percentage of the corresponding uninhibited control reaction. A Student's *t*-test was used to test for statistical significance.

Results

N-Dealkylation of perphenazine by cDNA-expressed CYP isoforms

Initially, we screened the cDNA-expressed CYP isoforms 1A2, 3A4, 2C8, 2C9, 2C18, 2C19, 2D6 and 2E1 for their ability to catalyse the N-dealkylation of perphenazine.

Only CYP2E1 did not display a measurable activity. The reaction rate of *N*-dealkylation as a function of the substrate concentration in the range 0–50 μM was measured, and the calculated K_m , V_{max} and CL_{int} (V_{max}/K_m) values are given in Table 2. For CYP1A2, CYP3A4 and CYP2D6 significantly better fits were obtained, when the Michaelis–Menten equation using two binding sites was used. The CL_{int} values point towards major contributions by CYP1A2, CYP3A4, CYP2C19 and CYP2D6, and Figure 2 displays the relation between reaction rate (V) and the ratio of reaction rate to substrate concentration (V/S) for these CYP isoforms (Eadie–Hofstee plots). The shapes of the plots for CYPs 1A2, 3A4, and 2D6 are in accordance with the presence of more than one binding site.

Specificity of CYP inhibitors

Initial inhibition experiments were carried out using cDNA-expressed CYP isoforms in order to determine inhibitor concentrations characterized by a high inhibition of the target CYP isoform and a reasonably low inhibitory effect towards the other CYP isoforms (Figure 3). A substrate concentration of 4 μM was used for all inhibitor experiments. Furafylline was added to the incubation medium in the concentration range 1.25–50 μM , and the inhibitory effect on the CYP1A2-mediated *N*-dealkylated perphenazine formation was measured. In order to obtain

more than 80% inhibition of the control reaction, a furafylline concentration of 50 μM was necessary, but concentrations exceeding 25 μM resulted in more than 20% inhibition of the CYP2C19-mediated reaction. No significant inhibition of CYP2D6 or CYP3A4 activities was found, when 25 μM furafylline was added to the medium. Thus, this concentration, which yielded 75% inhibition of CYP1A2, was chosen for inhibition of HLM-mediated reactions. In the same manner, ketocozazole was studied in the range 0.125 μM to 1 μM , and the optimum concentration was found to be 0.5 μM . At this concentration, CYP3A4 was inhibited by 98%, and the other reactions were either insignificantly inhibited or only slightly inhibited (13% as regards CYP2C19). For fluvoxamine, the range 0.125 μM to 5 μM was considered with selection of 0.25 μM as the optimum value. The target enzyme, CYP2C19, was only inhibited by 52% at this concentration, but an increase of the fluvoxamine concentration to more than 0.25 μM resulted in a too high inhibition of CYP1A2 and also led to significant inhibition of CYP3A4 and CYP2D6. Finally, the inhibitory effect of quinidine was tested over the range 0.125 μM to 10 μM , and 5 μM was selected as the optimum concentration for further experiments. This relatively high concentration was needed to attain 80% inhibition of CYP2D6 activity. CYP3A4 activity was inhibited by 16%, whereas that of the other two isoforms not were subject to significant inhibition by 5 μM quinidine.

Table 2 K_m , V_{max} and CL_{int} (V_{max}/K_m) for the *N*-dealkylation of perphenazine mediated by cDNA-expressed isoforms (mean with s.e. in parenthesis). The parameters were calculated on the basis of duplicate incubations using 5–9 different substrate concentrations.

	K_m (μM)	V_{max} ($\text{nmol h}^{-1} \text{nmol}^{-1} \text{CYP}$)	CL_{int} ($\text{ml h}^{-1} \text{nmol}^{-1} \text{CYP}$)
CYP1A2 *	0.87 (0.56)	44 (12)	50
	1.7×10^6 ★★	4.5×10^6 ★★	2.7
CYP3A4*	7.9 (0.56)	59 (12)	7.5
	0.82×10^3 ★★	3.2×10^3 ★★	3.9
CYP2D6 *	1.9 (1.0)	180 (67)	95
	62 (47)	750 (34)	12
CYP2C19***	14 (1.5)	750 (34)	54
CYP2C8	28 (12)	81 (16)	2.9
CYP2C9	53 (55)	38 (23)	0.72
CYP2C18	14 (8.0)	23 (4.6)	1.6

* Best fit was obtained using a two-site Michaelis–Menten function. ** s.e. could not be calculated. *** Value obtained from the highest substrate concentration omitted in the calculation.

Figure 2 Eadie-Hofstee plots displaying rates of perphenazine *N*-dealkylation (V) mediated by cDNA-expressed CYPs 1A2, 3A4, 2C19 and 2D6 as a function of the ratio between reaction rate and substrate concentration (V/S). The substrate concentration ranges from 0 to 50 μM . The last point in parenthesis was omitted when estimating the kinetic parameters for CYP2C19. Each symbol represents the mean of duplicate determinations from independent incubations.

N-Dealkylation of perphenazine by human liver microsomal preparations

The rate of *N*-dealkylated perphenazine formation at various substrate concentrations mediated by the HLM pool is shown in Figure 4 in the form of an Eadie-Hofstee plot. The shape of the plot may be interpreted as a significant involvement of several CYP isoforms with one exhibiting substrate inhibition.

The contributions of the individual CYP isoforms to perphenazine *N*-dealkylation were assessed by inhibition

experiments using the HLM pool and the three individual HLM preparations. The optimal inhibitor concentrations outlined above were used. Figure 5 presents the mean values for inhibition together with the quantitative amounts of the CYP isoforms in the various HLM preparations (see Table 1). Of the CYP2C family only CYP2C9 was quantified, but according to studies by other authors the mean catalytic activity of CYP2C19 amounts to about 25% of CYP2C9 activity [27]. The contribution of CYP1A2 was verified by a significant inhibition by furafylline in all but the HLM assigned HG112, which had

Figure 3 Inhibition of cDNA-expressed CYP isoform-mediated *N*-dealkylation of perphenazine. The substrate concentration was 4 μ M, and inhibition is expressed as a percentage of the rate of the control reaction without inhibitors. The bars and vertical lines (s.d.) represent the means of six inhibition experiments. □ furofylline 25 μ M; ▨ ketoconazole 0.5 μ M; ▤ fluvoxamine 0.25 μ M; ■ quinidine 5 μ M.

a very low CYP1A2 content. In the latter HLM, only 2.6% inhibition was observed. The involvement of CYP3A4 as measured by ketoconazole inhibition varied roughly in proportion to the CYP3A4 content of the four HLMs. In microsomes from liver HG112 fluvoxamine but not furofylline had a significant inhibitory effect on perphenazine metabolism, indicating the involvement of CYP2C19. Finally, the contribution of CYP2D6 is demonstrated by inhibition by quinidine, the extent of which was related to the amount isoform present. In microsomes from HG30 quinidine inhibited perphenazine *N*-dealkylation significantly by 13%, although the preparation did not contain a measurable amount of CYP2D6. This may be ascribed to a small degree of inhibition of CYP3A4 activity by quinidine (see Figure 3). Assuming H161Pool microsomes represent an average HLM, our data indicate that the mean contribution of CYP3A4 to perphenazine *N*-dealkylation is about 40% of total *N*-dealkylated perphenazine formed, and those of CYPs 1A2, 2C19 and 2D6 are 20–25% each. With respect to the three individual HLMs, the contribution of CYP2D6 varied from 13% to 44% and that of CYP3A4 from 14% to 89%.

Substrate consumption and *N*-dealkylated perphenazine formed

In order to investigate the importance of the *N*-dealkylation reaction as a function of the total metabolism of perphenazine by HLM, the amount of *N*-dealkylated perphenazine formed was compared with the decrease in

Figure 4 Perphenazine *N*-dealkylation mediated by a pooled human liver microsomal preparation (H161Pool). The data are presented as an Eadie-Hofstee plot showing the *N*-dealkylation rate (*V*) as a function of *V*/*S*, where *S* is the perphenazine concentration. Each data point represents the mean of duplicate determinations from independent incubations.

the substrate concentration after an incubation period of 10 min. The initial substrate concentration was 4 μ M, and 200 μ g protein of the H161Pool per assay was used. The mean substrate concentration decreased by 1.010 ± 0.322 μ M (s.d.; $n=12$), and the mean amount of *N*-dealkylated perphenazine formed was 0.577 ± 0.062 μ M. Thus, the *N*-dealkylation pathway represented 57% of the total perphenazine metabolism catalysed by microsomes from the H161Pool microsomes.

Discussion

Our studies using recombinant enzymes suggest that CYPs 1A2, 3A4, 2C19 and 2D6 can catalyse the *N*-dealkylation of perphenazine. Further work in HLM using selective inhibitors of individual CYPs show that most of the metabolite formation could be ascribed to the sum of the activities of these four isoforms. This interpretation of the results is based on the assumption that the inhibition patterns observed for the cDNA-expressed isoforms are also valid for these in HLM. The individual contributions of the four isoforms to *N*-dealkylated perphenazine formation may vary with the perphenazine concentration. The relatively high affinities and high capacities of CYP1A2 and CYP2D6 may result in higher relative contributions to total *N*-dealkylation by these two isoforms at lower substrate concentrations. The clinically relevant concentration range of perphenazine in human

Figure 5 Effect of selective CYP inhibitors on perphenazine N-dealkylation mediated by a pooled human liver microsomal preparation (H161Pool) and three individual liver microsomal preparations (HG23, HG30 and HG112). The substrate concentration was 4 μM, and inhibition is expressed as a percentage of the rates of reaction without inhibitors. The up-turned bars represent the mean of six inhibition experiments (s.d.). The CYP content of the four preparations, determined by the manufacturer using Western blotting, are also given (down-turned bars). The CYP2C19 contents are set to 25% of the CYP2C9 contents.

liver tissue is unknown, but *post mortem* analyses of perphenazine concentrations in humans, who had ingested an overdose of perphenazine, showed liver concentrations of the drug that were up to 50 times higher than those in blood [28, 29]. At steady-state, the usual trough plasma perphenazine concentration is in the range 2–6 nM, increasing to about 20 nM during the drug absorption phase [5, 7, 28–30]. Thus, clinically relevant hepatic concentrations are probably in the low micromolar range. The value of 4 μM was chosen as a compromise between the use of a relatively low perphenazine concentration and the achievement of a high analytical precision.

Chemical inhibitors may be more or less specific for their target CYP isoform depending among other factors on the concentration used (21–23). We thus evaluated the minimum required concentration for attaining a reasonably high inhibition of the target form in relation to *N*-dealkylation of perphenazine and the associated specificity of inhibition. Given a competitive pattern of inhibition, relatively high inhibitor concentrations are necessary to displace a substrate with a high affinity. Quinidine inhibition of *N*-dealkylation of perphenazine by CYP2D6 exemplifies this situation. Perphenazine is known to have a high affinity for CYP2D6 giving rise to important kinetic drug interactions, and accordingly the K_m value recorded here for the CYP2D6 mediated *N*-dealkylation was low (1.9 μM) [12, 13 31]. A relatively high quinidine concentration (5 μM) was necessary to achieve an inhibition of more than 80%. This is a quinidine concentration somewhat higher than that used by others. For example, inhibition of 1'-hydroxylation of bufuralol can be accomplished by concentrations below 1 μM [32]. Similarly, perphenazine exhibits a high affinity to CYP1A2 (K_m 0.87 μM), and it was necessary to apply a relatively high concentration of furafylline to achieve an acceptable degree of inhibition. Furafylline may be used either as a competitive inhibitor without preincubation or as a mechanism-based inhibitor requiring preincubation [23]. We used furafylline as a competitive inhibitor, because this approach is compatible with the supplier's recommended procedure of starting the reaction by addition of recombinant enzyme. Further, our preliminary investigations indicated that in agreement with other studies furafylline preincubation may lead to activation of CYP3A4 [33].

The mean steady-state plasma concentration/dose (C/D) ratio of patients lacking CYP2D6 (poor metabolizers) is about twice that of extensive metabolizers, and based on a population kinetic study the use of CYP2D6 genotype has been suggested for optimizing the dose of perphenazine in patients [34]. Our *in vitro* findings indicate that on average CYP2D6 is only responsible for about 25% of the *N*-dealkylated perphenazine formation in the liver, but the contribution of this isoform may vary considerably from

subject to subject because of quantitative variation in liver CYP2D6 content and the genetic polymorphism of CYP2D6. The relative abundance and catalytic activity of CYPs 1A2, 3A4 and 2C19, involved in the *N*-dealkylation of perphenazine, also displays large inter-individual variation [35].

The involvement of four isoforms in the *N*-dealkylated perphenazine formation may help to explain the large interindividual variation of the perphenazine C/D observed in patients treated with the drug. However, the modest role of CYP2D6 found in the present study does not explain that poor metabolizers have an average C/D-value at steady-state twice that of extensive metabolizers [16]. By measurement of substrate disappearance, we found that *N*-dealkylation amounted to only 57% of total perphenazine metabolism mediated by our pooled HLM preparation. Thus, it is likely that the other metabolic routes like sulfoxidation and ring-hydroxylation depend more strongly on CYP2D6.

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