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Oxidation of Histamine H1 Antagonist Mequitazine is Catalyzed by Cytochrome P450 2D6 in Human Liver Microsomes

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ABSTRACT

Mequitazine [10-(3-quinuclidinylmethyl) phenothiazine] is a long-acting and selective histamine H1-receptor antagonist that is mainly biotransformed by human liver microsomes to yield hydroxylated and S-oxidized metabolites. Mequitazine hydroxylase was inhibited by propranolol and quinidine. Lineweaver-Burk plots for the hydroxylation and the S-oxidation indicated that the hydroxylation occurred with a low K_m (0.72 ± .26 μM) in human liver microsomes. Microsomes from genetically engineered human B-lymphoblastoid cells expressing cytochrome P450 2D6 (CYP2D6) efficiently metabolized mequitazine to the hydroxylated and S-oxidized metabolites. The results indicate that CYP2D6 isozyme is a major form of CYP responsible for the metabolism of mequitazine in human liver microsomes. Inhibition of CYP3A-catalyzed midazolam 1′-hydroxylase by various histamine H1 antagonists, including mequitazine, suggested that mequitazine and some other histamine H1 antagonists could also be inhibitors of CYP3A in human liver microsomes.

Many isozymes of CYP have been identified, purified, cloned and expressed (Nelson *et al*. 1996). CYP consists of a superfamily of mixed-function monooxygenases associated with the metabolism of a broad group of structurally unrelated compounds, including drugs, chemical carcinogens, environmental pollutants and endogenous substrates such as steroids, fatty acids and prostaglandins (Guengerich, 1991). The intensity and duration of drug effects depend on the activity of CYP and other drug metabolizing enzymes in the liver. It is widely recognized that the individual variability of drug metabolism is caused, in part, by genetic polymorphism of CYPs including CYP2D6, known as the debrisoquine/sparteine-type genetic polymorphism (Eichelbaum and Gross, 1990). CYP2D6 has a role in the metabolism of a wide range of drugs, particularly antiarrhythmic agents, β-blocking agents, antidepressants and other clinically useful drugs (Eichelbaum and Gross, 1990). In poor metabolizers, the low capacity to metabolize drugs results in a crucial role in the incidence and spectrum of side effects seen in some patients. If poor metabolizers are treated with a drug having a narrow therapeutic index, that is a substrate for CYP2D6, an exaggerated response and/or serious and toxic effects are expected to occur at therapeutic doses as a result of increased plasma concentration of the parental active form. The large interindividual variability in plasma concentrations of several tricyclic antidepressants seen in patients treated with similar doses has been attributed mainly to impaired metabolic capacity of CYP2D6 (Brøsen *et al*., 1986, Dahl and Bertilsson, 1993). Similar responses occur in extensive metabolizers if they are simultaneously treated with a potent inhibitor that competes with a substrate for the active site of CYP2D6. For example, during treatment with quinidine, EMs show the phenotype of PMs (Brøsen *et al*., 1987). Similarly, coadministration of imipramine and chlorpheniramine enhances the effect of imipramine *in vivo* in rats by the same mechanism (Alhaide and Mustafa, 1989).

Mequitazine is a potent, nonsedative and long-acting histamine H1 antagonist proven to be a better therapeutic drug than other conventional antihistamines (Fujimura *et al*. 1981). Adequate metabolic studies to determine which CYP isozyme mainly catalyzes this drug have not been reported in humans. The oxidative metabolism of mequitazine was extensively studied in rats and dogs (Uzan *et al*., 1976). Its

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ABBREVIATIONS: CYP, cytochrome P450; FMO, flavin-containing monooxygenase; HPLC, high-performance liquid chromatography; PM, poor metabolizer; EM, extensive metabolizer.

primary metabolic pathways in rats consist of the aromatic hydroxylation of the phenothiazine structure, S-oxidation and N-oxidation of a side chain (Hojo *et al*., 1981, Soda *et al*., 1981). The oxidation of drugs having phenothiazine structure is generally known to be catalyzed by CYP and/or FMO (Cashman *et al*., 1993).

We investigated the effects of CYP inhibitors and anti-CYP antisera on the mequitazine metabolism to determine whether CYP is responsible for the metabolism of mequitazine in human liver microsomes. In addition, we have assessed the inhibitory effects of histamine H1 antagonists, including mequitazine, on the 1′-hydroxylase activity of midazolam in human liver microsomes. We found mequitazine as a novel substrate for CYP2D6 and exerting an inhibitory effect of CYP3A-mediated drug metabolism *in vitro*.

Methods

Materials. $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan), quinidine from Tokyo Kasei Kogyo (Tokyo, Japan), propranolol and 7-ethoxycoumarin from Aldrich Chemical (Milwaukee, WI), terfenadine from Sigma Chemical (St. Louis, MO), promethazine, phenacetin, coumarin and tolbutamide from Wako Pure Chemicals (Osaka, Japan). Sulfaphenazole was a kind gift from Ciba-Geigy Japan (Takarazuka, Japan), cyclosporine A from Sandoz Pharmaceuticals (Tokyo, Japan) and Azelastine, mequitazine and mequitazine metabolites from Asahi Chemical Industry (Tokyo, Japan), respectively. All other chemicals and solvents were of the highest grade commercially available.

Human liver microsomes. Human livers were obtained from patients after pathological examination of specimens isolated after death or during hepatic surgery. Surgeries were performed for the removal of metastatic liver tumors. The use of the human liver for these studies was approved by the Ethics Committee of Hokkaido University. Liver samples were stored at -80°C until use. Microsomes were prepared as described previously (Kamataki and Kitagawa, 1973). The microsomal protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Analytical procedures. Metabolites of mequitazine were determined by HPLC. A typical reaction mixture consisted of 50 μM mequitazine, 100 mM potassium-phosphate buffer (pH 7.4), .05 mM EDTA, an NADPH-generating system (0.5 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate and 1 U/ml of glucose 6-phosphate dehydrogenase) and human liver microsome (0.5 mg/ml) in a final volume of .25 ml. The reaction mixture was preincubated at 37°C for 5 min. Reaction was initiated by the addition of the NADPH-generating system. After incubation for 30 min, 0.25 ml of ice-cold acetonitrile was added to stop the reaction. Promethazine hydrochloride (160 ng dissolved in distilled water) was added to the sample as an internal standard. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant was directly subjected to HPLC equipped with a L-7100 pump (Hitachi, Tokyo, Japan), a SPD10A UV-Visible absorbance detector (Shimadzu, Kyoto, Japan), a C-R6A integrator (Shimadzu, Kyoto, Japan) and an analytical column Nova-Pak Phenyl (60 Å, 4 μm, 3.9 mm i.d.x 150 mm; Waters, Tokyo, Japan). Metabolites were separated using the following solvent system: 19% acetonitrile; 81% 0.047 M ammonium acetate (pH 5.5), 0 to 10 min; 19 to 37% linear gradient, 10 to 20 min; 37% acetonitrile 20 to 30 min; flow rate 1.0 ml/min. The elution of mequitazine and its metabolites was monitored at 250 nm.

Kinetics of mequitazine metabolism. Mequitazine metabolism proceeded linearly with incubation time up to 60 min when 50 μM of mequitazine and .5 mg of microsomal protein/ml were added to the incubation mixture. Metabolites of mequitazine were produced linearly with an incubation time of up to 60 min and with the amounts of microsomal protein of up to 1.0 mg/ml. Accordingly, incubations for the kinetic studies were carried out with 0.5 mg of microsomal protein/ml at 37°C for 50 min. In kinetic studies, three different human livers (HL12, HL29, HL35) were used. To determine kinetic parameters, the mequitazine concentration ranged from 1 to 50 μM. Linweaver-Burk plots and Eadie-Hofstee plots were used for the determination of kinetic parameters. The values were used to estimate apparent kinetic parameters by linear least-squares regression analysis.

Inhibition studies. To examine the possibility that the hydroxylation of mequitazine is catalyzed by a specific isozyme of CYP, the effects of inhibitors of CYP on the production of the hydroxylated metabolite were studied. A typical incubation mixture contained 100 mM potassium-phosphate (pH 7.4), .05 mM EDTA, an NADPH-generating system and 0.2 mg/ml microsomal protein. Phenacetin, caffeine, coumarin, 7-ethoxycoumarin, sulfaphenazole, tolbutamide, propranolol, chlorzoxazone or cyclosporine A was added to the incubation mixture as the relatively specific inhibitor of a CYP isozyme at a concentration of 100 μM. An inhibitor was added to the incubation mixture and preincubated for 5 min before the reaction was started with an NADPH-generating system. All inhibitors were dissolved in methanol and added to the incubation mixture at a final methanol concentration of 1%.

A typical incubation mixture for the assay of midazolam metabolism contained 100 mM potassium-phosphate (pH 7.4), 50 μM EDTA, 12.5 μM midazolam, an NADPH-generating system and 0.1 mg/ml of microsomal protein in a final volume of 0.25 ml. Azelastine, mequitazine, promethazine or terfenadine was added to the incubation mixture as an inhibitor of a CYP at a concentration of 12.5, 25 or 50 μM. The sample was subjected to the same HPLC systems as described above, except that an analytical column TSK-GEL ODS-120T (120 Å, 5 μm, 4.6 mm i.d.x 150 mm; Tosoh, Tokyo, Japan) was used. The mobile phase consisting of 10 mM sodium acetate (pH 7.8)/acetonitrile/methanol (53: 29: 18, v/v/v) was delivered at a flow rate of 1.0 ml/min. The elution of midazolam and its metabolites was monitored at 240 nm. Other methods were the same as described above. For immuno-inhibition studies, the preparations of rabbit anti-human CYP2D6 serum and rabbit anti-rat CYP3A2 serum from Daiichi Pure Chemicals (Tokyo, Japan) were used. Rabbit anti-human CYP2D6 recognized CYP2D6 and did not cross-react with human CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2E1 and 3A4 (N Shimada, unpublished data). The immuno-inhibition of mequitazine metabolism was examined by preincubation of human liver microsomes with various concentrations of anti-human CYP2D6 serum at room temperature for 30 min. Incubations were carried out as described above, except that microsomal protein 0.125 mg was added in a final volume of 0.25 ml.

Metabolism of mequitazine by human CYPs expressed in human B lymphoblastoid cells. Microsomes from human B-lymphoblastoid cells expressing human CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 were obtained from Daiichi Pure Chemicals (Tokyo, Japan). As a negative control, microsomes from cells carrying the expression vector without CYP cDNA were used. Incubations were carried out as described above, except that microsomal protein (0.125 mg) was added in a final volume of 0.2 ml. Other methods were the same as described in the analytical procedure.

Results

Metabolism of mequitazine in human liver microsomes. The proposed *in vitro* metabolic pathways of mequitazine in human liver microsomes are illustrated in figure 1. Mequitazine and its metabolites are well separated with HPLC. Typical HPLC chromatograms are illustrated in figure 2. Incubation of mequitazine with human liver microsomes yielded two major metabolites, both of which were produced linearly with an incubation time of up to 60 min

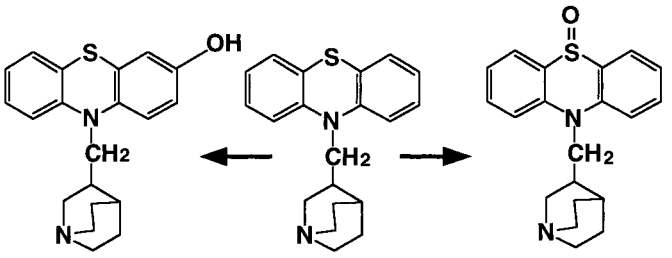

Fig. 1. Proposed *in vitro* major metabolic pathways of mequitazine in human liver microsomes.

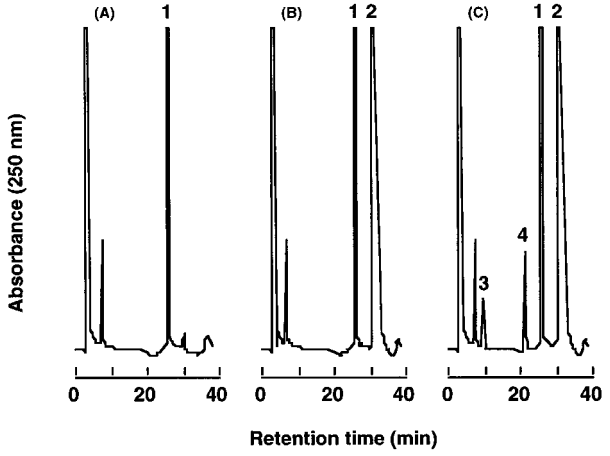

Fig. 2. Representative HPLC chromatograms of mequitazine and its metabolites produced by human liver microsomes. The elution pattern of the reaction mixture incubated without mequitazine is shown in A, the chromatogram from the reaction mixture without human liver microsomes is shown in (B) and the reaction mixture containing 0.5 mg/ml human liver microsomes and 50 μM mequitazine is shown (C). Liver sample HL12 was used. Peak identifications are as follows: (1) promethazine (25 min); (2) mequitazine (30 min); (3) S-oxidized mequitazine (10 min); (4) hydroxylated mequitazine (9.5 min).

and with the amounts of microsomal protein of up to 1.0 mg per incubation. Typical Lineweaver-Burk plots are shown in figure 3a. Thus, the apparent *CL*int value of the hydroxylation and the S-oxidation were estimated to be 38 and 4.5 μl/min/nmol P450 in human liver microsomes (HL12), respectively. Eadie-Hofstee plots for the hydroxylation of mequitazine by human liver microsomes (HL12, HL29 and HL35) are shown in figure 3b. A biphasic pattern in the hydroxylation was seen. These results suggest that at least two enzymes are involved in the hydroxylation. The kinetic parameters of hydroxylation for high- (K_m1, V_{max}1) and low- (K_m2, V_{max}2) affinity enzymes were calculated. Thus, apparent K_m1 was estimated to be .72 ± .26 μM with V_{max}1 of 17 ± 6.6 pmol/min/mg protein for the high-affinity enzyme. The apparent K_m2 was 4.7 ± 2.2 μM and V_{max}2 was 15 ± 8.8 pmol/min/mg protein for the low-affinity enzyme (mean ± S.D., n = 3).

Effects of CYP inhibitors on the hydroxylation of mequitazine. To clarify the forms of CYP responsible for the hydroxylation of mequitazine, the effects of inhibitors on the activity of mequitazine hydroxylase were examined (fig. 4). Specific inhibitors of CYPs employed in the present study included phenacetin (an inhibitor of CYP1A1 and CYP1A2) (Tassaneeyakul *et al*., 1993), caffeine (CYP1A2) (Tassaneeyakul *et al*., 1994), coumarin (CYP2A6) (Yamano *et al*., 1990),

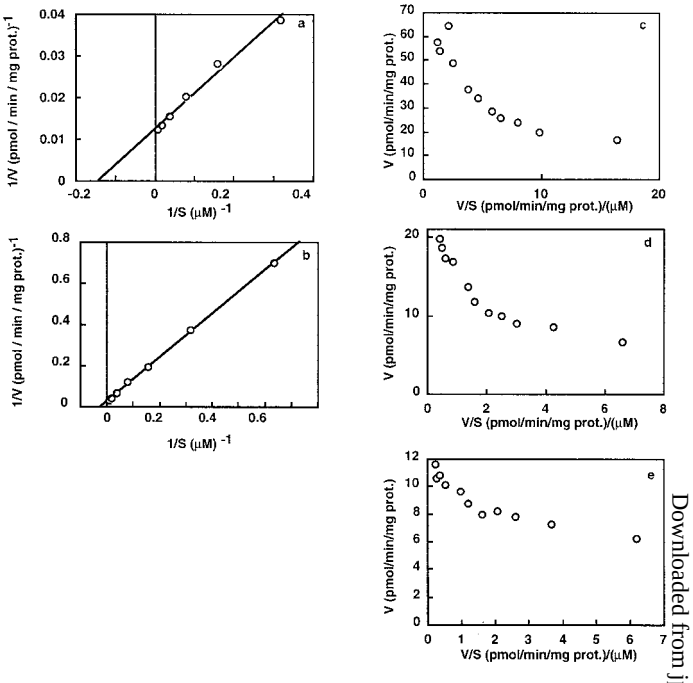

Fig. 3. a and b, Representative Lineweaver-Burk plots for mequitazine metabolism. (a; hydroxylation, B; S-oxidation) by human liver microsomes (HL12). A reaction mixture (250 μl) contained 0.5 mg/ml human liver microsomes, 100 mM potassium-phosphate buffer (pH 7.4), .05 mM EDTA, an NADPH-generating system and mequitazine (1–50 μM). This mixture was incubated at 37°C for 30 min. c-e, Eadie-Hofstee plots for mequitazine hydroxylation by three preparations of human liver microsomes (a, HL12; b, HL29 and c, HL35). The specific contents of P450 in liver microsomes from HL12, HL29 and HL35 were 524, 275 and 162 pmol/mg protein, respectively.

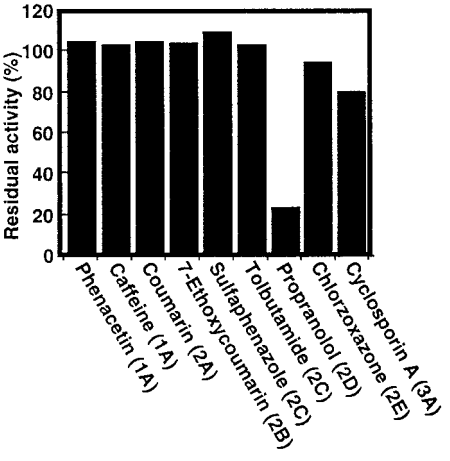

Fig. 4. Effects of CYP inhibitors on the hydroxylation of mequitazine. A panel of inhibitors selective for a particular isozyme of human CYP was used at a 100 μM concentration with microsomes from HL12. The inhibitor is shown on the bottom of the graph. Mequitazine was used at a concentration of 10 μM. The rate of the hydroxylation in the absence of an inhibitor was 44 pmol/min/mg prot. Data are the mean value from the duplicate determinations.

7-ethoxycoumarin (CYP2B6) (Baker *et al*., 1995), sulfaphenazole, tolbutamide (CYP2C8, CYP2C9) (Rettie *et al*., 1992), propranolol (CYP2D6) (Distlerath *et al*., 1985), chlorzoxazone (CYP2E1) (Hyland *et al*., 1992) and cyclosporin A (CYP3A) (Kronbach *et al*., 1988; Fischer *et al*., 1994). Among these inhibitors, propranolol inhibited the mequitazine hydroxylase potently (about 80% inhibition), although cyclospo-

rin A inhibited weakly (about 20% inhibition). These results suggest that the mequitazine hydroxylation was mainly catalyzed by CYP2D6.

To further confirm the involvement of CYP2D6 in the mequitazine hydroxylation, quinidine, a known typical and potent inhibitor of CYP2D6 (Otton *et al.*, 1984; Rodrigues 1994) was added to the reaction mixture (fig. 5). As a result, mequitazine hydroxylation was suppressed by the inhibitor in a concentration-dependent manner. These results further support the idea that CYP2D6 is the major enzyme that catalyzes the hydroxylation of mequitazine in human livers.

Immuno-inhibition by rabbit anti-human CYP2D6 serum of the metabolism of mequitazine in human liver microsomes. To confirm the involvement of CYP2D6 in the hydroxylation of mequitazine, an immuno-inhibition study was performed using rabbit anti-human CYP2D6 antibodies (fig. 6). Rabbit anti-human CYP2D6 antibodies inhibited the mequitazine hydroxylase in liver microsomes from the human subject of HL12. At the highest concentration of the antibodies, the hydroxylation of mequitazine was inhibited by 90%. This result is consistent with the results of the effects of inhibitors and lends support to the idea that CYP2D6 is mainly involved in the hydroxylation of mequitazine in human liver microsomes.

Metabolism of mequitazine by CYPs expressed in microsomes of human B-lymphoblastoid cells. Metabolism of mequitazine by microsomes from human B lymphoblastoid cells expressing human CYPs (CYP1A1, 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4) was investigated (fig. 7). The hydroxylation was exclusively catalyzed by CYP2D6. These results further support the notion that CYP2D6 is the key enzyme that catalyzes the metabolism of mequitazine in human livers.

Effects of histamine H1 antagonists on the catalytic activities of CYP3A4. Biotransformation of the histamine H1 antagonist terfenadine is well known to be mediated specifically by CYP3A4 in human liver microsomes. Thus, terfenadine was expected to be an inhibitor of CYP3A. Together with this possibility, it was examined whether or not other histamine H1 antagonists, including mequitazine, were capable of inhibiting the CYP3A-mediated drug metabolism, adopting midazolam as a substrate. Midazolam 1′-hydroxylation is a highly selective CYP3A reaction (Gorski *et al.*, 1994). As shown in figure 8, midazolam 1′-hydroxylase was inhibited by histamine H1

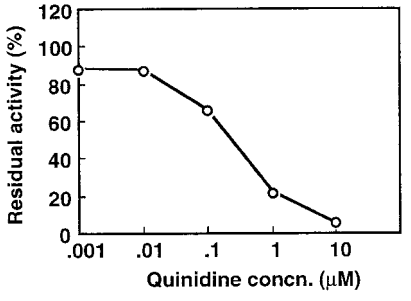

Fig. 5. Inhibition by quinidine of the mequitazine metabolism by human liver microsomes. Mequitazine was used at a concentration of 10 μM. Liver microsomes from a human subject (HL12) were used for the mequitazine hydroxylation. The rate of the hydroxylation in the absence of an inhibitor was 44 pmol/min/mg prot. Incubations were performed in duplicate.

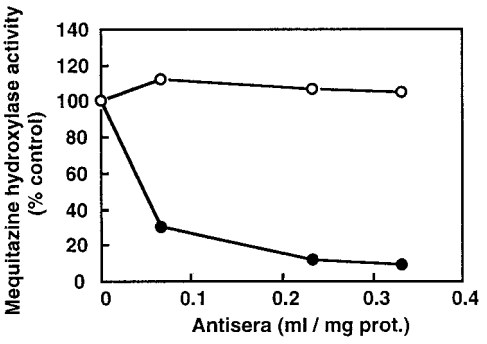

Fig. 6. Immuno-inhibition by anti-human CYP2D6 serum of the hydroxylation of mequitazine by human liver microsomes. Mequitazine was used at a concentration of 10 μM. Microsomes (HL12) were preincubated in the presence of indicated amounts of rabbit anti-human CYP2D6 serum (●) and rabbit preimmune serum (○) at room temperature for 30 min. Mequitazine was used at a concentration of 10 μM. The rate of the hydroxylation in the absence of an inhibitor was 56 pmol/min/mg prot. Each datapoint represents the mean of duplicate determinations.

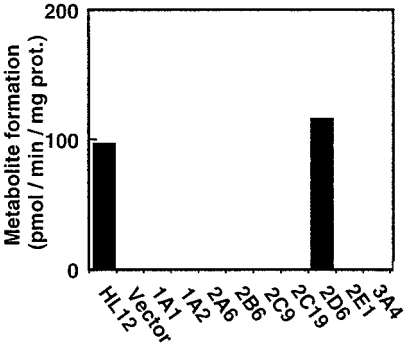

Fig. 7. Metabolism of mequitazine by microsomes from genetically engineered B-lymphoblastoid cells expressing a specific human CYP. Microsomes (HL12) were preincubated in the presence of an NADPH-generating system for 5 min. Mequitazine was used at 5 μM concentration. The hydroxylated metabolite of mequitazine was determined. Each data point represents the mean of duplicate determinations.

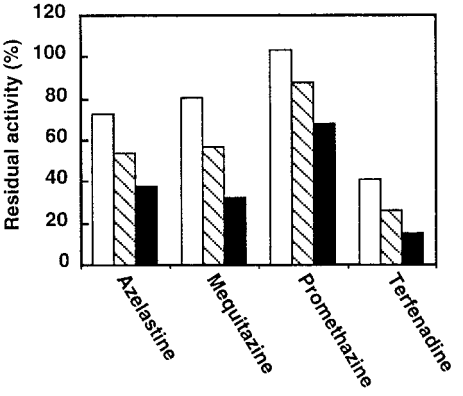

Fig. 8. Inhibitory effects of histamine H1 antagonists on midazolam 1′-hydroxylase activity in human liver microsomes. The reaction mixture contained human liver microsomes (HL12) (0.5 mg/ml), 100 mM potassium-phosphate buffer (pH 7.4), 0.05 mM EDTA and 25 μM midazolam. Inhibitor was added to the incubation mixture at a concentration of 12.5 (open column), 25 (hatched column) and 50 μM (closed column). The reaction was started by an NADPH-generating system after 5 min preincubation at 37°C. Each data point represents the mean of duplicate determinations.

antagonists used in our study. The kinetic values for the inhibition by the histamine H1 antagonists in human liver microsomes are shown in Table 1. The K_i values of these

TABLE 1
K_i values for the inhibition of midazolam 1′-hydroxylase in human liver microsomes by three histamine H1 antagonists

Inhibitor	Type of Inhibition	Apparent K_i (μM) (n = 5, mean ± S.E.)
Mequitazine	Mixed	45.9 ± 19.6
Azelastine	Mixed	14.5 ± 2.7
Terfenadine	Mixed	14.6 ± 4.2

drugs showing potent inhibition were estimated by Lineweaver-Burk plots (Cornish-Bowden and Eisenthal, 1978). The K_i values (mean ± SD, n = 5) of mequitazine, azelastine, and terfenadine for the midazolam 1′-hydroxylase were 45.9 ± 19.6, 14.5 ± 2.7 and 14.6 ± 4.2 μM, respectively.

Discussion

This study was originated with a sparteine PM subject who exhibited serious sedation by taking histamine H1 antagonist. This PM subject had a new mutation in the *CYP2D6* gene designated as *CYP2D6*18* (Yokoi *et al*., 1996). Phenotyping studies with debrisoquine, sparteine and metoprolol have shown that the expression of human CYP2D6 is polymorphic in a number of racial groups, and approximately .84% of Japanese are PMs (Nakamura *et al*., 1985; Ishizaki *et al*., 1987; Sohn *et al*., 1991). Mequitazine has been marketed in Japan and several European countries for the treatment of bronchial asthma and allergic manifestations. Mequitazine, as with other structurally related phenothiazines, is extensively metabolized in mammalian species. Studies conducted with human liver microsomes have shown that mequitazine was primarily converted to S-oxidized and hydroxylated compounds. This was in agreement with the results of *in vivo* metabolic studies performed by analysis of human urine (data not shown). Judging from the fact that the V_{max}/K_m of mequitazine hydroxylation was higher than that of the S-oxidation, this pathway was assumed to play a major role in the metabolism of mequitazine. In our study, the *in vitro* formation of hydroxylated metabolites from mequitazine by human liver microsomes was strongly inhibited by a well-known CYP2D6 inhibitor, quinidine. Although the amount of CYP2D6 in human liver microsomes was estimated to be only about 5 pmol/mg of microsomal protein, or about 1.5% of total CYP (Shimada *et al*., 1994), CYP2D6 was found to be the enzyme mainly involved in the hydroxylation of mequitazine in our study. These findings suggest that mequitazine is likely to interact with human CYP2D6 *in vivo* with the potential of causing drug interaction. Among other histamine H1 receptor antagonists, mepyramine was reported to have a high affinity for human CYP2D6 (Hiroi *et al*., 1995) and the metabolism of loratadine was also partially inhibited by quinidine (Yumibe *et al*., 1995). CYP2D6 was proven in our study to play a major role in mequitazine metabolism, and the plasma concentration of mequitazine in PM subjects might reach a level higher than was expected even after the administration of therapeutic doses. Because histamine H1 antagonists are widely used in medication for the common cold, it can be a new phenotyping drug to judge CYP2D6 PM.

Peak serum concentration of the single 5-mg oral dose of mequitazine in humans was 3.19 ± 1.70 ng/ml (9.89 ± 5.27 nM) (Ylitalo *et al*., 1989), and this value was approximately three orders lower than the inhibition constants for CYP3A mediated-midazolam 1′-hydroxylation by histamine H1 antagonists, azelastine, mequitazine and terfenadine. This makes it unlikely that histamine H1 antagonist mequitazine would cause drug interactions on CYP3A-metabolizing drugs, since plasma concentrations of mequitazine are quite low. Because CYP2D6 was proven in our study to play a major role in mequitazine metabolism, the plasma concentration of mequitazine in CYP2D6 PM subjects might reach a level higher than was expected even after administration of therapeutic doses.

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