IN VITRO IDENTIFICATION OF THE HUMAN CYTOCHROME P-450 ENZYMES INVOLVED IN THE N-DEMETHYLATION OF AZELASTINE

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ABSTRACT:
Azelastine hydrochloride [4-[4-chlorophenyl)methyl]-2-(hexahy-dro-1-methyl-1H-azepin-4yl)-1-(2H)-phthalazinone monohydrochloride], is a long-acting antiallergic and antiamthmetic drug. The human cytochrome P-450 (CYP) isoform responsible for azelastine N-demethylation, the major metabolic pathway for azelastine, has been examined. Eadie-Hofstee plots of azelastine N-demethylation in human liver microsomes were biphasic. In microsomes from baculovirus-infected insect cells, recombinant CYP3A4, 2D6, 1A2, and 2C19 exhibited high azelastine N-demethylation activity. The $K_v$ values of the recombinant CYP2D6 (3.75 $\mu$M) and CYP3A4 (43.7 $\mu$M) were relatively close to that of high-affinity (14.1 $\mu$M) and low-affinity (54.7 $\mu$M) components in human liver microsomes, respectively. Azelastine N-demethylation activity was inhibited only by the anti-CYP3A antibody, in contrast to antibodies for CYP1A2, 2D6, and 2C. In addition, desmethylazelastine formation was significantly inhibited by ketoconazole and troleandomycin but only weakly by omeprazole, sulfaphenazole, and furafylline. These observations suggested that the N-demethylation of azelastine is most extensively catalyzed by the CYP2D6 and 3A4 isoforms in humans.

Azelastine hydrochloride [4-[4-chlorophenyl)methyl]-2-(hexahy-dro-1-methyl-1H-azepin-4yl)-1-(2H)-phthalazinone monohydrochloride] is a long-acting antiallergic and antiamthmetic drug (McTavish and Sorkin, 1989; Chand et al., 1990; Richards et al., 1990), the properties of which extend beyond histamine $H_1$ receptor-blocking activity. These include antagonism of the chemical mediators adenosine, leukotriene C$_4$, leukotriene D$_4$, endothelin-1, and platelet activation factor and the inhibition of the generation and/or release of histamine, interleukin-1$,\beta$, and superoxide free radicals. It has been reported that azelastine is metabolized to desmethylazelastine (Tatsumi et al., 1984), which is pharmacologically equivalent to azelastine (Perhach et al., 1989; Szelényi, 1989). Desmethylazelastine (Fig. 1), which is produced from azelastine by cytochrome P-450 (CYP)$^1$ in human liver, shows a plasma concentration of ~50% of the azelastine levels at the steady state after the oral administration of azelastine hydrochloride (Morganroth et al., 1997). Furthermore, desmethylazelastine is distributed at a much higher concentration than azelastine in guinea pig lung, which is a pharmacologically targeted tissue after oral administration (Chand et al., 1993). The possibility that the alteration of the extent of N-demethylation of azelastine could correspond to individual variations in pharmacological activity cannot, at present, be excluded. The prediction of drug interactions and other environmental and genetic factors that influence the metabolism of azelastine requires the identification of the specific CYP isoform(s) that are responsible for N-demethylation. Morganroth et al. (1997) showed that azelastine has no effect on CYP3A4 activity but inhibits CYP2D6 and 2C19 activities with $K_v$ values in excess of the maximum plasma concentrations by 120- to 800-fold, and that desmethylazelastine inhibits only CYP2C19 with a $K_v$ value equivalent to that of azelastine. This group further demonstrated that azelastine did not induce adverse effects resulting from excessive H$_1$-antagonist activity with the coadministration of ketoconazole for 14 days in healthy volunteers. However, the specific isoforms responsible for the formation of desmethylazelastine have not been rigorously identified.

The objective of this study was to identify the CYP isoform(s) that are specifically involved in azelastine N-demethylation in humans to clarify the possible drug interactions of azelastine.

Materials and Methods
Chemicals and Biochemicals. Azelastine and desmethylazelastine were synthesized at Eisai Co. Ltd. (Tokyo, Japan). Troleandomycin, quinidine, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). $\beta$-NADPH was obtained from Oriental Yeast (Tokyo, Japan). 4-Methylpyrazole and coumarin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Omeprazole was supplied by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Other chemicals were of the highest grade commercially available. Furafylline; ketoconazole; rabbit anti-human CYP1A1/1A2, CYP2D6, and CYP3A4 serum; and goat anti-human CYP2C serum were purchased from Daiichi Pure Chemicals (Tokyo, Japan). A mixed pool of human liver microsomes from 10 male subjects was obtained from International Institute for the Advancement of Medicine (Exton, PA). The microsome pools were analyzed in 1997 to determine the enzyme activity of CYP isoforms that are expressed in human liver (CYP1A2, CYP2A6, CYP2C, CYP2D, CYP2E, CYP3A, and CYP4A). Microsomes from baculovirus-infected insect cells expressing different human CYP isoforms and control microsomes that had not been transfected were obtained from Gentest Corp. (Woburn, MA).

Incubation Conditions. All incubations with respect to liver microsomes were carried out at a protein concentration of 0.5 mg/ml in 100 mM potassium phosphate buffer (pH 7.4) including 0.1 mM EDTA and the NADPH-gener-
Chemical Inhibition. Inhibition experiments were carried out at an azelastine concentration near the apparent \( K_m \) of the high-affinity reaction for formation of desmethylazelastine (15 \( \mu \)M) in a final volume of 0.5 ml for 5 min. With the exception of furafylline, the various inhibitors were dissolved in ethanol and then added in 10-\( \mu \)l aliquots (final concentration, 0.5% v/v) to the microsomes. Furafylline was dissolved in dimethyl sulfoxide (DMSO), and 10-\( \mu \)l portions were then added. A 10-\( \mu \)l aliquot of ethanol or DMSO was used in control experiments. The stock solutions of inhibitors were added just before the addition of azelastine. In the case of the mechanism-based inhibitors, furafylline and troleandomycin, a mixture of inhibitor, microsomes, and the NADPH-generating system was preincubated for 10 min at 37°C before the addition of azelastine. Desmethylazelastine formation in the presence of inhibitors was compared with appropriate controls (no preincubation or 10-min preincubation), and the results were calculated as a percentage of the uninhibited rate.

Immunoinhibition by Anti-CYP Antiserum. Different concentrations of anti-CYP antiserum and microsomes (0.5 mg/ml) were incubated for 30 min at room temperature. A solution of azelastine (final concentration, 50 \( \mu \)M) was then added, and the mixture was maintained at 37°C for 1 min. The reaction (5 min) was initiated via the addition of the NADPH-generating system. The results were calculated as a percentage of the duplicate control measurements.

Metabolism of Azelastine by Microsomes from Human CYP-Expressed Baculovirus-Infected Insect Cells. This experiment involved the use of microsomes from baculovirus-infected insect cells expressing CYP1A2, 2A6, 2C9-Arg144, 2C9-Cys144, 2D6, 2E1, 3A4, or 3A5. Control microsomes were obtained from baculovirus-infected insect cells that had not been transfected. All CYPs used were coexpressed (from cDNA) with NADPH-CYP reductase. In addition, CYP3A4 and CYP2E1 were also coexpressed with cytochrome b5. The final concentrations of microsomes and azelastine used were 5 to 50 pmol CYP/mg and 5 to 100 \( \mu \)M, respectively. A 30-min reaction at 37°C was initiated by adding the NADPH-generating system.

HPLC Analysis. The determination of azelastine and desmethylazelastine was performed with a Hitachi HPLC system (L6000 pump, L-7480 fluorescence detector, and D-2500 Chromato-integrator, Tokyo, Japan). An aliquot of sample was injected onto a LiChrospher 100 CN (5 \( \mu \)m) column (4.5 inside diameter \( \times \) 250 mm, Cica-MERCK, Darmstadt, Germany), using a 20-\( \mu \)l loop to achieve high precision in the injection volume of 20 \( \mu \)l and eluted with 0.025% diethylamine in methanol at a flow rate of 1 ml/min. Fluorescence detection was at 280 nm for excitation and 366 nm for emission. The detection limit for desmethylazelastine was 20 nM in the case of the incubation sample.

A calibration curve obtained from 0.1 to 4 \( \mu \)M was constructed by plotting desmethylazelastine concentration versus the peak area of desmethylazelastine (\( r = 0.998 \)). The coefficient of variation and relative error in range of 0.1 to 4 \( \mu \)M for desmethylazelastine were 1.8 to 4.5% and 2.3 to 9.5%, respectively.

Data Analysis. Experimental reaction velocity measurements were combined to provide mean \( \pm \) S.D. values. The parameters \( K_m \) and \( V_{max} \) were estimated by fitting the Michaelis-Menten equation to the data using nonlinear regression analysis (MULTI, Yamaoka et al., 1981) with weighted data (1/\( y^2 \)). Initial estimates for nonlinear regression were chosen based on substrate concentration (S) versus reaction velocity (V) and Eadie-Hofstee plots (V/S versus V).

Results

Metabolism of Azelastine in Human Liver Microsomes. Azelastine and desmethylazelastine were chromatographically resolved from the other endogenous components (Fig. 2). Incubation of azelastine with human liver microsomes yielded only one peak with a retention time identical with that of desmethylazelastine. In addition, the concentration of azelastine decreased, and a corresponding increase in the concentration of desmethylazelastine was observed, indicating that only desmethylazelastine is produced as the result of in vitro metabolism in human liver microsomes. The formation of desmethylazelastine from 50 \( \mu \)M azelastine increased with incubation times up to 60 min. On the other hand, the rate of formation was constant at the initial stage of the reaction but subsequently became slower with time. To ensure a constant reaction rate for azelastine \( N \)-demethylation, an incubation time of 5 min was used. Figure 3 depicts an Eadie-Hofstee plot of the formation of desmethylazelastine from azelastine in pooled human liver microsomes. The biphasic nature of the plot indicates contributions of multiple enzymes for the \( N \)-demethylation reaction. The calculated values of the high-affinity component, \( K_{m1} \) and \( V_{max1} \), were 14.1 \( \pm \) 1.38 \( \mu \)M and 0.615 \( \pm \) 0.048 nmol/min/mg, respectively. In the low-affinity component, \( K_{m2} \) was 54.7 \( \pm \) 2.63 \( \mu \)M, and \( V_{max2} \) was 1.29 \( \pm \) 0.025 nmol/min/mg.

Inhibition Analysis. To determine the specific CYP isozyme(s) involved in the biotransformation, incubations were conducted using chemical inhibitors that are specific for various CYP isozymes (Fig. 4). Ketoconazole (Baldwin et al., 1995) and troleandomycin (Chang et al., 1994) significantly inhibited the \( N \)-demethylation of azelastine to \(~20\%\) of the control values, even at low concentrations (10 \( \mu \)M).
Data are expressed as the averages (± S.D.) of triplicate determinations. In contrast, antihuman CYP2C and 70% at 2.5 mg IgG/nmol CYP, whereas antihuman CYP2D6 antibody showed only weak inhibition. In contrast, antihuman CYP2C and

Fig. 3. Eadie-Hofstee plot for azelastine N-demethylase activity in pooled human liver microsomes. Human liver microsomes (0.5 mg/ml) were incubated with azelastine (3–150 μM), and the rates of formation of desmethylazelastine were determined by HPLC. Data are expressed as the averages (± S.D.) of triplicate determinations.

Fig. 4. Inhibition of azelastine N-demethylase activity by CYP inhibitors in human liver microsomes at an azelastine concentration of 15 μM. The range of inhibitor concentrations was 10 to 100 μM. Furafylline ( ), CYP1A2, coumarin ( ), CYP2A6, sulfaphenazole ( , CYP2C9), omeprazole ( , CYP2C19), quinidine ( , CYP2D6), 4-methylpyrazole ( , CYP2E1), ketocazole ( , CYP3A4), and troleandomycin ( , CYP3A4) were used. The activity in the absence of an inhibitor was 0.27 and 0.21 nmol/min/mg, respectively, using ethanol and DMSO as solvents for preparation of inhibitor stock solutions. Each data point represents the mean (± S.D.) of triplicate determinations.

Omeprazole (an inhibitor of 2C19; Ko et al., 1997) and quinidine (an inhibitor of CYP2D6; Otton et al., 1988) inhibited azelastine N-demethylation in a dose-dependent manner. Furafylline (an inhibitor of CYP1A2; Sesardic et al., 1990) and sulfaphenazole (an inhibitor of CYP2C9; Baldwin et al., 1995) inhibited the N-demethylation only at high concentrations (100 μM). Coumarin (an inhibitor of CYP 2A6; Yamano et al., 1990) and 4-methylpyrazole (an inhibitor of CYP2E1; Newton et al., 1995) had no effect on N-demethylation activity. These data suggest that CYP3A4 is the primary isomor involved in the N-demethylation of azelastine. To further confirm the specific CYP isozyme(s) involved in azelastine metabolism, an immunoinhibition study was performed using antihuman CYP antibodies. As shown in Fig. 5, antihuman CYP3A antibody inhibited N-demethylation by 70% at 2.5 mg IgG/nmol CYP, whereas antihuman CYP2D6 antibody showed only weak inhibition. In contrast, antihuman CYP2C and

Fig. 5. Effects of anti-CYP antibodies on azelastine N-demethylation in human liver microsomes. Azelastine (50 μM) was incubated with human liver microsomes (0.5 mg/ml) in the presence of antihuman CYP antibodies for 5 min at 37°C after preincubation of each antibody with liver microsomes for 30 min at room temperature. Values represent the means of duplicate measurements as a percentage of duplicate control measurements, 0.41 nmol/min/mg.

1A1/IA2 antibodies showed no effect on the N-demethylation reaction.

Azelastine N-Demethylation Activity by Microsomes from Human CYPs Expressed in Baculovirus-Infected Insect Cells. The metabolism of azelastine by microsomes from baculovirus-infected insect cells expressing human CYPs (CYP1A2, 2A6, 2C9Arg, 2C9Cys, 2C19, 2D6, 2E1, 3A4, or 3A5) was investigated (Fig. 6). The activities of CYP1A2, 2C19, 2D6, and 3A4 without cytochrome b5 and 3A5 were 4.18 ± 0.185, 2.34 ± 0.255, 2.33 ± 0.148, 2.28 ± 0.077, and 0.907 ± 0.114 pmol/min/pmol CYP, respectively. CYP2A6, 2C9Arg, 2C9Cys, and 2E1 showed an extremely low activity for N-demethylation whereas CYP 3A4, coexpressed with cytochrome b5, showed the highest activity for formation of desmethylazelastine. The kinetic parameters for recombinant CYP1A2, 2C19, 2D6, and 3A4 with cytochrome b5 are listed in Table 1. CYP2D6 showed a very small K_m and the highest V_max/K_m value, indicating that CYP2D6 contributes to the high-affinity component in human liver microsomes. CYP3A4, coexpressed with cytochrome b5, showed the highest V_max value among recombinant CYPs (17.6 ± 0.41 pmol/min/pmol CYP), and its K_m was 43.7 ± 4.05 μM, which was close to the K_m value of the low-affinity component in human liver microsomes. CYP1A2 and 2C19 showed relatively low V_max/K_m values as compared with CYP2D6 and 3A4.

Discussion

A number of approaches have been developed recently for the identification of human CYPs responsible for the metabolism of various drugs (Wrighton et al., 1993; Miners et al., 1994; Rodrigues, 1994). The identification of CYPs for metabolism of drugs is available for the prediction of drug-drug interactions in clinical use. Neither azelastine nor its metabolite have been reported to induce drug-drug interactions and/or toxicity, such as cardiotoxicity. In contrast, another H1 receptor antagonist, terfenadine, which is also metabolized by CYP3A4, showed an increase in its plasma concentration and a prolongation of the Q-T interval when CYP3A4 was inhibited by ketoconazole (Wrighton et al., 1993; Honig et al., 1993). It has been reported that azelastine is metabolized primarily to desmethylazelastine, which is the only metabolite detectable in human plasma after...
administration. The pharmacological activity of desmethylazelastine is equivalent in potency to azelastine. The plasma concentrations of desmethylazelastine were 2.6 ± 1.2 ng/ml for C_{min} and 3.4 ± 1.3 ng/ml for C_{max} after the administration of 4 mg of azelastine twice per day for 14 days in healthy volunteers, compared with a C_{min} of 3.8 ± 2.7 ng/ml and a C_{max} of 6.33 ± 3.5 ng/ml for azelastine (Morganroth et al., 1997). Desmethylazelastine is more easily taken up by the lung as a target tissue than is the parent azelastine (Chand et al., 1993). Therefore, the extent of metabolism of azelastine to desmethylazelastine might be of some importance, in terms of pharmacological activity. However, the identification of enzymes for the metabolism of azelastine has not been investigated in detail. In this study, using several complementary techniques, the CYP isoforms involved in the formation of the major human liver microsomal metabolite of azelastine have been identified.

Only desmethylazelastine is formed when azelastine is incubated with human liver microsomes; this confirms that desmethylazelastine is a major metabolite in humans. Furthermore, Edde-Hofsteet plots indicate that the metabolism of azelastine is catalyzed by multiple enzymes. Inhibition studies using ketoconazole, troleandomycin, and the highly specific anti-CYP3A antibody, strongly suggest that the formation of desmethylazelastine is predominantly catalyzed by CYP3A. Furthermore, the azelastine N-demethylation activity in microsomes, which express individual CYP isoforms, was highest for CYP3A4 coexpressing cytochrome b5, among the several recombinant CYPs (Fig. 6). Cytochrome b5 has been found to be required for optimal CYP3A activity, but its effect appears to be dependent on the particular CYP3A substrate (Gillam et al., 1995; Yamazaki et al., 1996a,b). Although the effects of the addition of cytochrome b5, in a CYP reconstitution system, on the K_{m} and V_{max} values for the N-demethylation of azelastine were not clarified in this study, coexpressed cytochrome b5 plays an important role in the electron transfer process in CYP3A catalyzed N-demethylation of azelastine in human liver. In addition, the activity with respect to N-demethylation of azelastine in human recombinant CYPs is 3-fold less for CYP3A5 than for CYP3A4. The presence of CYP3A in human liver has been reported to be ~29% of the total CYP content (Shimada et al., 1994). The expression of CYP3A5, as determined by immunoblot analysis, was found in only 29% of all human livers analyzed and is approximately 10 to 30% of the relative amount of CYP3A4 when expressed (Wrighton et al., 1990). Because of the polymorphic expression of CYP3A5, it appears that this isoform does not substantially contribute to the metabolism of azelastine. Moreover, the K_{m} value in CYP3A4-expressing microsomes (43.7 μM) was close to that of the low-affinity component (54.7 μM) in human liver microsomes. The lack of, or very weak, inhibition of azelastine metabolism by anti-CYP2D6, 2C, and 1A antibodies suggests that CYP3A4 can efficiently catalyze N-demethylation when other CYP isoforms are inhibited by antibody. These findings suggest that CYP3A4 is the primary isoform of low-affinity component and that it is a major contributor of the N-demethylation of azelastine.

Although a lack of inhibition by anti-CYP1A2, 2D6, and 2C antibodies was observed, N-demethylation was moderately inhibited by omeprazole and quinidine and marginally so by furafylline and sulfaphenazole (Fig. 4). Moreover, recombinant CYP1A2, 2C19, and 2D6 exhibited an activity similar to that of CYP3A4 without coexpressed cytochrome b5, as shown in Fig. 6. CYP1A2 showed the lowest intrinsic clearance for the N-demethylation of azelastine because it had the highest K_{m} value, despite a high V_{max} value. Therefore, CYP1A2 might be less responsible for N-demethylation of azelastine in human liver at a low concentration of azelastine after oral clinical dose. Interestingly, CYP2D6 showed a low K_{m}, a low V_{max}, and the highest clearance (V_{max}/K_{m}) values. The intrinsic clearance of CYP2C19 was 3.4-fold less than that of CYP2D6. The content of CYP2C has been reported to constitute ~25% of the CYP isoforms expressed in human liver microsomes (Shimada et al., 1994). In addition, 60% of the CYP2C cDNA clones isolated from a human liver library were CYP2C9, and only 1% were CYP2C19 (Romkes et al., 1991). These findings suggest that the contribution of CYP2C19 to azelastine N-demethylation activity in human liver microsomes is negligible. Kinetic analyses suggest that CYP2D6 contributes predominantly to the high-affinity component of azelastine metabolism in human liver microsomes. The inhibitory effect of omeprazole in the formation of desmethylazelastine was not only on CYP2C19 but also CYP3A, because omeprazole is a known substrate for these isozymes (Ko et al., 1997).

Clinical studies have demonstrated that individuals with reduced CYP2D6-mediated metabolism represent a high-risk group with a propensity to develop adverse drug effects (Smith, 1986). Among other H₁ receptor antagonists, mequitazine and mepyramine have been reported to have a high affinity for human CYP2D6 (Hiroi et al., 1995; Nakamura et al., 1998). In contrast, azelastine N-demethylation is mainly catalyzed by CYP2D6 and 3A4, indicating that this reaction can be catalyzed by CYP3A4 when CYP2D6 is inhibited by coadministered drugs. CYP3A is the major CYP subfamily in the human liver and is involved in the metabolism of a wide variety of pharmaceutical drugs that are metabolized by CYP. In addition, CYP3A enzymes have been reported to be involved in interactions with several drugs such as macrolides, ketoconazole, and cyclosporin (Picard et al., 1990; Periti et al., 1992). A single oral dose of 2 mg of
azelastine given to healthy volunteers produced mean maximum plasma concentrations of 1.88 nM (Tokuanga and Iijima, 1993), which is much smaller than the $K_{m}$ value in liver microsomes. The low dose of azelastine and the large amount of CYP3A in the human liver make it less likely that clinically significant drug-drug interactions would occur with CYP3A substrates. In fact, Morganroth et al. (1997) reported that the coadministration of azelastine and ketotifenazol failed to produce any electrocardiographic effect. In contrast, the possibility of clinical drug interactions at the enzyme level might exist if azelastine is administered at the same time as a drug that is preferentially metabolized by CYP2D6. However, it has been reported that the $K_{m}$ values for the inhibition of (S)-metropolol (a substrate for CYP2D6 metabolism) by azelastine, which range from 1.7 to 12.1 μM in human liver microsomes, are substantially higher than those that are likely to be encountered therapeutically (Morganroth et al., 1997). Therefore, the potential for the presence of azelastine to result in a decrease in the clearance of drugs metabolized by CYP2D6 would be expected to be small and not clinically detectable.

Moreover, the lung, a target organ for azelastine, is capable of the $N$-demethylation of azelastine. It has been reported that there are multiple forms of P-450 in human lung microsomes (Shimada et al., 1995). Therefore, the potential for the presence of azelastine to result in a decrease in the clearance of drugs metabolized by CYP2D6 would be expected to be small and not clinically detectable.

In conclusion, azelastine is metabolized only to desmethylazelastine in humans, and the conversion is primarily catalyzed by CYP2D6 and 3A4. Moreover, treatment of patients with azelastine is not expected to result in significant pharmacokinetic interactions.

References


