EFFECTS OF OLOPATADINE, A NEW ANTIALLERGIC AGENT, ON HUMAN LIVER MICROSONAL CYTOCHROME P450 ACTIVITIES

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ABSTRACT:

Olopatadine, a new histamine H\(_1\) receptor-selective antagonist, is a tricyclic drug containing an alkylamino moiety. Some compounds containing a similar alkylamino group form a cytochrome P450 (P450) -iron (II)-nitrosoalkane metabolite complex [metabolic intermediate complex (MIC)], thereby causing quasi-reversible inhibition of the P450. There was concern that olopatadine might also form MICs, therefore, the present investigation was undertaken to explore this possibility. We identified the enzymes catalyzing olopatadine metabolism and investigated the effect of olopatadine on human P450 activities. During incubation with human liver microsomes in the presence of a NADPH-generating system, olopatadine was metabolized to two metabolites, M1 (N-monodemethyl-ethylolopatadine) and M3 (olopatadine N-oxide) at rates of 0.330 and 2.50 pmol/min/mg protein, respectively. Troleandomycin and ketoconazole, which are both selective inhibitors of CYP3A, significantly reduced M1 formation but specific inhibitors of other P450 isoforms did not decrease M1 formation. Incubation of olopatadine with cDNA-expressed human P450 450 isoforms confirmed that M1 formation was almost exclusively catalyzed by CYP3A4. The formation of M3 was enhanced by N-0ctylamine and was inhibited by thiourea. High specific activity of M3 formation was exhibited by cDNA-expressed flavin-containing monoxygenase (FMO1) and FMO3. Olopatadine did not inhibit P450 activities when it was simultaneously incubated with substrates for different P450 isoforms. Also, P450 activities in human liver microsomes were unaffected by pretreatment with olopatadine or M1. Furthermore, spectral analysis revealed that neither olopatadine nor M1 formed an MIC. Therefore, it is unlikely that olopatadine will cause drug-drug interactions involving P450 isoforms.

1 Abbreviations used are: P450, cytochrome P450; MIC, metabolic intermediate complex; HPLC, high-performance liquid chromatography; FMO, flavin-containing monoxygenase; LC/MS/MS, high-performance liquid chromatography with tandem mass spectrometry; ESI, electrospray ionization; SKF-525A, Proadifen.

Olopatadine, (Z)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenzo[b,e]oxepin-2-oxide) at rates of 0.330 and 2.50 pmol/min/mg protein, respectively. Troleandomycin and ketoconazole, which are both selective inhibitors of CYP3A, significantly reduced M1 formation but specific inhibitors of other P450 isoforms did not decrease M1 formation. Incubation of olopatadine with cDNA-expressed human P450 450 isoforms confirmed that M1 formation was almost exclusively catalyzed by CYP3A4. The formation of M3 was enhanced by N-0ctylamine and was inhibited by thiourea. High specific activity of M3 formation was exhibited by cDNA-expressed flavin-containing monoxygenase (FMO1) and FMO3. Olopatadine did not inhibit P450 activities when it was simultaneously incubated with substrates for different P450 isoforms. Also, P450 activities in human liver microsomes were unaffected by pretreatment with olopatadine or M1. Furthermore, spectral analysis revealed that neither olopatadine nor M1 formed an MIC. Therefore, it is unlikely that olopatadine will cause drug-drug interactions involving P450 isoforms.

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investigating MIC formation by olopatadine. Therefore in this study, we have explored the possibility of drug-drug interactions involving olopatadine via MIC formation using in vitro techniques. We have 1) examined the conditions under which olopatadine is N-dealkylated to a primary amine by human liver microsomes (formation of a primary amine is hypothesized to be the first step for MIC formation); 2) identified the enzymes catalyzing olopatadine metabolism; 3) investigated the effects of olopatadine on P450-mediated reactions; and 4) monitored spectral changes that occurred during metabolism of olopatadine by liver microsomes (in general, the formation of MIC exhibits a peak at approximately 454 nm in the absorption spectrum).

**Materials and Methods**

**Chemicals.** Olopatadine, reference M1, M2, and M3 were synthesized in our institute. \[^{14}C\]Olopatadine (2.18 GBq/mmol) was synthesized at Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK) and the purity was more than 99% pure as ascertained by high-performance liquid chromatography (HPLC). Ketoconazole was supplied by Janssen Pharmaceuticals (Beerse, Belgium). Other chemicals were obtained from the following sources: 4-acetylaminophenol and \[^{14}C\]Olopatadine by liver microsomes (in general, the formation of MIC is hypothesized to be the first step for MIC formation); 2) identified the enzymes catalyzing olopatadine metabolism; 3) investigated the effects of olopatadine on P450-mediated reactions; and 4) monitored spectral changes that occurred during metabolism of olopatadine by liver microsomes (in general, the formation of MIC exhibits a peak at approximately 454 nm in the absorption spectrum).

**Proposed metabolic pathway of olopatadine.**

**Fig. 1.** Proposed metabolic pathway of olopatadine.
scale incubation (12.5 ml) using a Bond Elute C18 cartridge (Varian, Harbor City, CA).

For metabolism by recombinant P450 and FMO expression systems, [14C]olopatadine (10 μM, 6.7 kBq/ml) or olopatadine (10 μM) was incubated with a NADPH-generating system in phosphate buffer, pH 7.4, at 37°C. Reactions were initiated by addition of microsomes of recombinant P450 or FMO (1 mg protein/ml) at 37°C. All incubations were conducted in duplicate and the duplicate values agreed within 85 to 115% of each other; the results are presented as the mean of the duplicate data.

**Effects of Enzyme Inhibitors and Activators on the Formation of M1 and M3.** To determine the effect of compounds on the metabolism of olopatadine. [14C]olopatadine (10 μM, 6.7 kBq/ml), enhancers or inhibitors, microsomes (1 mg/ml), and NADPH-generating system were mixed and incubated at 37°C for 1 h in a final volume of 100 μl. In the cases of furafylline, diethyldithiocarbamate, troleandomycin, SKF-525A, N-ocytamine, and thiourea, the mixture of microsomes and these compounds was preincubated in the presence of a NADPH-generating system at 37°C for 15 min and then the reaction was initiated by addition of [14C]olopatadine. The concentration of added compounds was 10 μM except for N-ocytamine, which was added at 100 μM. All inhibitors were dissolved in methanol; the final concentration of methanol was 1.0 vol% in the reaction mixture.

**Effects of Olopatadine on P450 Isozyme-Specific Activities.** The effect of olopatadine on the activity of all P450 enzymes was examined using two different incubation methods. A pretreatment method was used to estimate the metabolism-dependent inhibition, e.g., suicidal inhibition, and a simultaneous incubation method was used to assess metabolism-independent inhibition (e.g., competitive inhibition). The following activities were measured for the respective P450 isozymes: phenacetin O-deethylation (CFP1A2: phenacetin concentration; 100 μM), tolbutamide methylhydroxylation for CYP2C8/9 (tolbutamide concentration; 500 μM), S-mephenytoin 4′-hydroxylation for CYP2C19 (S-mephenytoin concentration; 200 μM), bufuralol 1′-hydroxylation for CYP2D6 (bufuralol concentration; 100 μM), chlorozoxazone 6-hydroxylation for CYP2E1 (chlorozoxazone concentration; 500 μM), and testosterone 6β-hydroxylation for CYP3A4 (testosterone concentration; 250 μM). In the preincubation method, incubation of olopatadine was carried out in the presence of the NADPH-generating system at 37°C for 30 min before the addition of the specific probe substrates. In the simultaneous incubation method, olopatadine was added with the specific probe substrates to the microsomal incubation mixture and then the reaction was initiated by addition of the NADPH-generating system. In these studies, higher concentrations of the P450-selective substrates than the respective Km values were used and, therefore, metabolism of the substrate should not have caused a significant alteration of the reaction rate (Boobis et al., 1998; Pelkonen et al., 1998; Ikeda et al., 2001).

**Analytical Methods. Analysis of Olopatadine and Its Metabolites.** Olopatadine and its metabolites were separated by HPLC using an instrument purchased from Hitachi (Tokyo, Japan); radioactivity was detected off line with a scintillation counter (Tri-Carb 2200CA or 2700; Packard Instrument Company, Inc., Meriden, CT). Reversed phase chromatography was carried out on a YMC-Pack AM312 (150 μm, 6.7 kBq/ml) or olopatadine (10 μM) was incubated with a NADPH-generating system in phosphate buffer, pH 7.4, at 37°C. Reactions were initiated by addition of microsomes of recombinant P450 or FMO (1 mg protein/ml) at 37°C. All incubations were conducted in duplicate and the duplicate values agreed within 85 to 115% of each other; the results are presented as the mean of the duplicate data.

The reaction mixture contained phosphate buffer (100 mM, pH 7.4), 1 mM EDTA, 10 mM MgCl2, and 1 mg of microsomal protein at 37°C. Olopatadine, M1, imipramine, or desipramine, dissolved in ethanol, were added to the test cuvette, and the same volume of ethanol was added to the reference cuvette. After the baseline was corrected, the NADPH-generating system was added to both cuvettes and the final volume adjusted to 1 ml, and then the difference spectra from 380 to 500 nm were recorded during incubation at 37°C at the designated time points using a 150-20 spectrophotometer (Hitachi).

**Results**

**Olopatadine Metabolism.** Two different metabolites, M1 and M3, were formed when olopatadine was incubated with human liver microsomes in the presence of an NADPH-generating system: after 1-h incubation, M1 and M3 accounted for 5.2 and 30.5% of the initial olopatadine, respectively. Structural information for these metabolites was obtained by LC/MS/MS analysis with electrospray ionization (ESI). The protonated molecular ion ([M + H]+) of M1 was observed at m/z 324 in the positive ESI spectrum, and fragment ions were detected at m/z 247 and 165 (Fig. 2A). The protonated molecular ion ([M + H]+) of M3 was observed at m/z 354 in the positive ESI spectrum, and fragment ions were detected at m/z 247 and 165 (Fig. 2B). The protonated molecular ion ([M + H]+) of olopatadine was observed at m/z 338 in the positive ESI spectrum, and fragment ions were detected at m/z 247 and 165 (Fig. 2C). From these data, M1 and M3 were identified tentatively as N-monodemethylolopatadine and olopatadine N-oxide, respectively, and this assignment was confirmed by demonstrating that these metabolites had identical retention times and mass spectra to the respective authentic standards. The formation of both M1 and M3 by human liver microsomes was found to be NADPH-dependent, and the formation rate of M1 and M3 was 0.330 and 2.50 pmol/min/mg protein, respectively. Kinetic parameters could not be calculated for the metabolites, because only low amounts of the metabolites were formed. In addition, M3 was reduced to olopatadine during incubation with human liver microsomes in the presence of NADPH under aerobic condition (data not shown). Therefore, the kinetic parameters could not be calculated accurately.

Selective inhibitors of P450 isozymes were used in this study to determine the potential roles of P450 isozymes in the formation of M1 and M3 (Fig. 3). SKF-525A, a nonspecific inhibitor of P450, and both troleandomycin and ketoconazole, which are inhibitors of CYP3A, markedly inhibited M1 formation. Selective inhibitors of other P450 enzymes, furafylline, sulfaphenazole, tranylcypromine, quinidine, and diethyldithiocarbamate, had little effect on M1 formation. However, none of the P450 inhibitors had significant effects on the formation of M3. In addition, the effects of N-ocytamine and thiourea on the formation of M1 and M3 were examined (Fig. 3). It has been reported that N-ocytamine inhibits P450 activity (Hodgson and Levi, 1998) and enhances FMO activity (McManus et al., 1987) and that thiourea inhibits the activity of P450 enzymes (Ortiz de Montellano and Correia, 1995) and FMO (Cashman et al., 1993). Although thiourea is a substrate of FMO, the clearance of thiourea by FMO is low (Kim and Ziegler, 2000) and, therefore, this should not compromise its use as an inhibitor in the present study. M1 formation was inhibited by N-ocytamine and thiourea but M3 formation was enhanced by N-ocytamine and inhibited by thiourea, suggesting that M3 was formed by FMO.

There was little difference in M1 formation by human liver microsomes treated without and with mild heat (45°C for 2 min); however, mild heat treatment of microsomes markedly lowered M3 formation (Fig. 3). Because, in contrast to P450, FMO is heat-labile (Cashman et al., 1993), these data provide further evidence that M3 formation is catalyzed by FMO.
The formation of M1 and M3 catalyzed by specific human P450 isozymes and FMO is shown in Fig. 4. The velocity of M1 formation by CYP3A4 was 0.00242 nmol/min/nmol P450. CYP1A2 generated small amounts of M1, but CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and FMO did not catalyze M1 formation. The velocities of M3 formation by FMO1 and FMO3 were 0.815 and 0.215 pmol/min/mg protein, respectively. The velocities of M3 formation by CYP1A2, CYP2B6, and CYP2E1 were less than one-fourth of the rate catalyzed by FMO3. The other P450 isozymes, CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, did not form M3.

Effects of Olopatadine, M1, and M3 on P450 Activities. Simultaneous addition of substrate and olopatadine (100 μM) did not affect the activities of CYP1A2 (phenacetin O-deethylation), CYP2C8/9 (tolbutamide methylhydroxylation), CYP2C19 (S-mephenytoin 4′-hydroxylation), CYP2D6 (bufuralol 1′-hydroxylation), CYP2E1 (chlorozoxazone 6-hydroxylation), and CYP3A4 (testosterone 6β-hydroxylation) (Fig. 5). However, specific P450 inhibitors inhibited the relevant P450 enzyme-selective activity under the incubation conditions used (Fig. 5).

Furthermore, the activity of each P450 isozyme was not affected significantly when human liver microsomes were pretreated with olopatadine, or 100 μM M1 and a NADPH-generating system (Fig. 6). However, imipramine weakly inhibited the activities of CYP2D6 and CYP3A, and desipramine inhibited the activities of CYP2C8/9, CYP2C19, CYP2D6, and CYP3A under the same conditions. In addition, although incubations of rat liver microsomes with desipramine in the presence of NADPH produced a change in the absorption spectrum at a wavelength of 455 nm (Fig. 7D), which was presumed to be due to the formation of MICs, neither olopatadine nor M1 altered the absorption spectrum (Fig. 7, A and B). Imipramine also did not affect the spectrum significantly (Fig. 7C), presumably because formation of desipramine was not sufficient under the experimental conditions.

Discussion

Many alkylamine derivatives are converted to reactive species that produce inhibitory complexes with P450 isozymes during microsomal P450-mediated metabolism. Because the first report of this phenomenon with SKF-525A (Schenkman et al., 1972), it has been demonstrated that amphetamine, macrolide antibiotics such as erythromycin and troleandomycin (Delaforge et al., 1983; Pessayre et al., 1983), and antihistamines such as diphenhydramine and orphenadrine also form complexes with P450 isozymes (Bast and Noordhoek, 1982; Bast et al., 1984). Similarly tricyclic antidepressants, such as imipramine, desipramine, and nortriptyline, inhibit P450 activity by the formation of an MIC (Murray and Field, 1992). Recent evidence also suggests that pharmacokinetic drug-drug interactions with clinical significance may be due, in part, to MIC of the inhibitor with P450 isozymes causing a decrease of P450 activity. The inhibitory effect seems to be relatively selective for the corresponding P450 isozyme(s); for exam-
Liver microsomes (1 mg of protein) were incubated for 60 min at 37°C with olopatadine and other specified compounds in the presence of an NADPH-generating system in a final volume 0.2 ml. For metabolism-based inhibitors or enhancers (i.e., SKF-525A, furafylline, diethyldithiocarbamate, troleandomycin, and N-octylamine), the microsomes were preincubated with these compounds for 15 min at 37°C in the presence of an NADPH-generating system before the addition of olopatadine. The concentrations of added inhibitors were 10μM except for N-octylamine, which was added at 100μM. The column labeled “Heat-treatment” represents the result obtained when the microsomes were heated at 45°C for 2 min. Activities are expressed as a percentage relative to the control experiment. The control activities of M1 and M3 formation were 0.330 and 2.50 pmol/min/mg protein, respectively. Each column represents the mean of two incubations. N.D., not detected.

When olopatadine was metabolized by human liver microsomes in the presence of a NADPH-generating system, two metabolites were formed: N-monodemethylolopatadine M1 and an N-oxide M3. It is noteworthy that N-demethylation seems to be an important first step for the formation of MIC (Bensoussan et al., 1995). However, only low amounts of M1 were generated from olopatadine in human liver microsomes. After oral administration to human subjects, the major metabolites of olopatadine in plasma and urine were reported to be M1 and M3, but the concentrations of these metabolites were much lower than that of unchanged drug (Tsunoo et al., 1995; Fujita et al., 1999). Thus, the low level of olopatadine metabolism observed in vitro is consistent with the low amount of olopatadine metabolites formed in vivo.

N-Dealkylation can be catalyzed by different P450 isozymes. For example, N-dealkylation of several drugs, including alfentanil (Yun et al., 1992) and amiodarone (Trivier et al., 1993), is catalyzed by CYP3A isozymes in human; a limited number of drugs, including amiflamine (Alvan et al., 1984) and citalopram (Gram et al., 1993) are N-dealkylated by CYP2D6; other isozymes that catalyze N-dealkylation in human are CYP1A2 (caffeine; Butler et al., 1989) and CYP2C19 (diazepam; Bertilsson et al., 1989).

In addition, drugs containing nitrogen can be metabolized via N-oxidation. The oxidation of nitrogen atoms is often catalyzed by P450 isozymes and FMO. In the present study, two approaches were used to identify the drug-metabolizing enzymes responsible for the formation of M1 and M3, namely, chemical inhibition and formation of MIC. Therefore, we investigated the effects of olopatadine on P450 activities in human liver microsomes to assess the potential for drug-drug interactions between olopatadine and other drugs.

Liver microsomes (1 mg of protein) were incubated for 60 min at 37°C with olopatadine and other specified compounds in the presence of an NADPH-generating system. Each column represents the mean of two incubations. N.D., not detected.
using two different incubation methods and a spectroscopic study. The two different incubation methods involved 1) preincubation and 2) simultaneous incubation with olopatadine and human liver microsomes in the presence of an NADPH-generating system. Metabolism-based inhibitors, such as furafylline and sorivudine (Ito et al., 1998), irreversibly bind to the enzyme, forming an MIC, and reduce both the activity and amount of the target enzymes. Thus, when metabolism-based inhibitors are incubated with target enzymes plus relevant cofactors before the addition of substrates, enzyme activity is inhibited strongly. On the other hand, when reversible (competitive) inhibitors are incubated simultaneously with target enzyme, cofactors, and substrates, enzyme activities are moderately inhibited. Because olopatadine and M1 did not inhibit P450 activities under either of the incubation conditions used, it was concluded that the compounds were not important inhibitors of P450 enzymes.

The above-mentioned conclusion was supported by the finding that
M1 did not produce a change in the optical difference spectrum in rat liver microsomes, although a positive control, desipramine, did produce a time-dependent change. When desipramine was incubated with rat liver microsomes, the spectrum shifted clearly at 454 nm, which was presumed to be due to the formation of an MIC. However, neither N-dealkylated olopatadine, M1, nor olopatadine itself caused a change in the optical difference spectrum in rat liver microsomes. These results suggest that the primary amine M1 does not form an MIC.

In conclusion, the present study indicates that olopatadine has no inhibitory effects on drug metabolism involving CYP1A2, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. Lack of any inhibitory effects is probably not surprising because olopatadine is poorly metabolized both in vitro and in vivo; the main elimination pathway is via urinary excretion of unchanged compound. Because the metabolic clearance of olopatadine in humans is very low, concurrent administration of inhibitors of metabolism is unlikely to alter
the pharmacokinetics of olopatadine significantly. Therefore, we conclude that drug-drug metabolic interactions involving MIC phenom-

enon by olopatadine are unlikely in clinical use.

References


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