**N-DEALKYLATION AND HYDROXYLATION OF EBASTINE BY HUMAN LIVER CYTOCHROME P450**

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**ABSTRACT:**

Ebastine [4-tert-butyl-4-[4-(diphenylmethoxy)piperidino]butyrophenone] is a new-generation, nonsedative, H₁ antihistamine. The present study was performed to characterize the cytochrome P450 (CYP) isoforms responsible for ebastine N-dealkylation and hydroxylation. Human liver microsomes metabolized ebastine to two major metabolites, i.e., a desbutyrophenone metabolite (des-BP) and hydroxyebastine (M-OH), and the ratio of Vₘₐₓ values was 3:1. N-Dealkylation yielded des-BP, whereas M-OH, an hydroxylation product, could be further oxidized to the pharmacologically active carebastine. In a panel of 14 human liver microsomal preparations, the rate of dealkylation showed a highly significant correlation with CYP3A-mediated testosterone 6β-hydroxylation but not with reactions of seven other CYP isoforms. However, there was no correlation between the two pathways for ebastine (dealkylation and hydroxylation). Differential chemical inhibition in liver microsomes, in which dealkylation was more sensitive than hydroxylation, was demonstrated with ketoconazole, troleandomycin, cyclosporin A, and midazolam. Anti-CYP3A antibodies markedly reduced the dealkylation rate (>95%) in liver microsomes but exhibited insignificant effects on hydroxylation (<5%). Among 12 cDNA-expressed human CYP isoforms, which account for up to 70% of the total CYP enzyme content in human liver, CYP3A4 alone metabolized ebastine; the ratio of des-BP to M-OH formation was 12:1. This ratio for metabolism by the pure enzyme was much larger than the ratio (3:1) observed for the microsomal reaction mixture. This change in ratio, which is attributed to a decrease in M-OH formation, indicates that, although ebastine is metabolized to two major metabolites, N-dealkylation to des-BP is mediated by CYP3A, whereas hydroxylation to M-OH appears to be mediated mainly by unidentified enzymes other than CYP3A.

The selective peripheral H₁ receptor antagonist ebastine [4-tert-butyl-4-[4-(diphenylmethoxy)piperidino]butyrophenone, CAS 90729–43-4] (fig. 1) belongs to a new generation of antihistamines characterized by negligible anticholinergic and antiserotonergic properties (Llupia et al., 1992) and the lack of sedative effects (Vincent et al., 1988a; Martinez-Toled et al., 1992; Hopes et al., 1992; Yakuo et al., 1994), which first-generation drugs such as diphenhydramine, promethazine, chlorpheniramine, and hydroxyzine exhibit (Kontou-Fili, 1994). Ebastine is further characterized by its lack of adverse cardiovascular effects, such as QT prolongation or torsades de pointes, which have been reported for the clinical use of terfenadine, another new-generation antihistamine, under certain conditions (Vincent et al., 1988b; Geary et al., 1995; Wiseman and Faulds, 1996; Monahan et al., 1990; Honig et al., 1992, 1993a,b). Ebastine has been shown to be effective for treatment of allergic rhinitis or chronic idiopathic urticaria, with only single daily doses (Kukita et al., 1994). After administration of oral doses of ebastine to experimental animals and human subjects, the agent was well absorbed and underwent virtually complete first-pass metabolism to its pharmacologically active, acid metabolite carebastine [4-[4-(4-diphenylmethoxy-1-piperidinyl)-1-oxobutyl]-α,α-dimethylbenzeneacetic acid, CAS 90729–42-3] (fig. 1) and other (inactive) metabolites (Fuji et al., 1994; Yamaguchi et al., 1994). The unchanged form was virtually absent from plasma at clinical doses (Yamaguchi et al., 1994).

To characterize the CYP¹ isoforms responsible for ebastine metabolism, the present study focused on the first steps of the pathway, i.e.
hydroxylation and N-dealkylation, using the following: 1) microsomes from four human livers possessing ample CYP-mediated monoxygenase activities, 2) a panel of microsomes from 14 human livers with predetermined activities for eight CYP isozyme substrates, 3) 12 cDNA-expressed CYP isoforms, 4) various CYP isozyme-selective inhibitors, and 5) anti-CYP isoform antibodies. The findings for hydroxylation were unexpected, refuting the previously accepted concept that the first step is catalyzed exclusively by CYP3A4 (Stevens et al., 1993).

Materials and Methods

Chemicals. 14C-labeled ebastine [4'-tert-butyl-4-[4-[(8-hydroxy-1,1-dimethylethyl)-4-[4-(diphenylmethoxy)piperidino]butyrophenone, lot S0604] was synthesized as described previously (Fuji et al., 1994), with a specific activity of 1.08 MBq/mg (29.2 µCi/mg) and radiochemical purity of >99%. Carebastine (lot PQ 1/89), 4-(diphenylmethylene)piperidine (desbutyrophenone, metabolite, des-BO; lot V-1002), and 4'-[2-hydroxy-1,1-dimethylheptyl]-4-[4-(diphenylmethoxy)piperidino]butyrophonone (hydroxy metabolite of ebastine, M-OH; batch 1, ref. 00244), as authentic metabolites, were supplied under license contract by Almirall-Prodesfarma S.A. (Barcelona, Spain). Furafylline, sulfaphenazole, and ketocanazole were purchased from Salford Ultrafine Chemical and Research Ltd. (Manchester, England). Courmarin, tranylcypromine, quinidine, diethyldithiocarbamate, TAPO, orphenadrine, and lauric acid were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade commercially available.

Human Liver Microsomes. Microsomal preparations from human liver samples obtained from kidney transplant donors, the relevant details for whom have been published elsewhere (Campbell et al., 1987), were characterized previously (Inaba et al., 1988; Tyndale et al., 1989). In brief, the preparations (coded as K14, K19, K20, and K27) have been demonstrated to have ample CYP2A2, CYP2E1, CYP2C9, and CYP3A4 activities.

Ebastine Metabolism by Human Liver Microsomes. Microsomes (0.1–1.0 mg protein/ml) were incubated with 2–30 µM [14C]ebastine for 0–60 min at 37°C, in 0.5-ml reaction mixtures containing 0.1 M phosphate buffer (pH 7.4), 1.0 mM NADPH, and 5.0 mM MgCl₂. The reactions were started by the addition of [14C]ebastine and stopped by the addition of methanol (1.0 ml). After centrifugation at 800g for 15 min, aliquots of the supernatants were evaporated to dryness under N₂ gas, and the residues were dissolved in methanol for analysis by TLC. The TLC plates used were prechanneled silica gel plates with preadsorbent spotting areas (J.T. Baker, Inc., Phillipsburg, NJ). The solvent system consisted of chloroform/methanol/ethyl acetate/acetic acid/water, 240:40:40:12:1 (v/v). After development, the TLC plates were exposed to phosphorimagering plates for 15–16 hr. The imaging plates were scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the quantified data were obtained using ImageQuant software (Molecular Dynamics).

Correlation Analyses. The HepatoScreen test kit (Human Biologics Inc., Phoenix, AZ) was used for the correlation experiments. The 14 different CYP inhibitors or substrates were used: 50 µM furafylline as a CYP1A2 inhibitor (Seradici et al., 1990; Kunze and Trager, 1993), 500 µM coumarin as a CYP2A6 competitive inhibitor (Yum et al., 1991; Nunoya et al., 1996), 50 µM orphenadrine as a CYP2B6 inhibitor (Heyn et al., 1996; Reidy et al., 1989), 10 µM sulfaphenazole as a CYP2C8/9/10 inhibitor (Back et al., 1988; Baldwin et al., 1995; Newton et al., 1995), 20 µM tranylcypromine as a CYP2C19 inhibitor (Inaba et al., 1985), 10 µM quinidine as a CYP2D6 inhibitor (Newton et al., 1995; Nunoya et al., 1996), 50 µM diethylthiocarbamate as a CYP2E1 inhibitor (Newton et al., 1995; Guengerich et al., 1991; Chang et al., 1994), 100 µM lauric acid as a CYP4A competitive inhibitor (Kawashima et al., 1994), 100 µM TA0 as a CYP3A inhibitor (Newton et al., 1995; Chang et al., 1994; Pessaye et al., 1983), 0.1–100 µM ketoconazole as a CYP3A inhibitor (at low concentrations) (Newton et al., 1995; Inaba et al., 1985), 50 µM diethylthiocolbamate as a CYP2E1 inhibitor (Newton et al., 1995; Guengerich et al., 1991; Chang et al., 1994), 100 µM lauric acid as a CYP4A competitive inhibitor (Kawashima et al., 1994), 100 µM TA0 as a CYP3A inhibitor (Newton et al., 1995; Chang et al., 1994; Pessaye et al., 1983), 0.1–100 µM ketoconazole as a CYP3A3 inhibitor (at low concentrations) and a nonspecific CYP inhibitor (at high concentrations) (Back et al., 1988; Baldwin et al., 1995; Newton et al., 1995; Maurie et al., 1992), and 0.1–100 µM cyclosporin A and midazolam (0.1–100 µM) as CYP3A competitive inhibitors (Kronbach et al., 1988, 1989). The concentrations of inhibitors/substrates described above were chosen based on the published K_{eq}, K_{m}, or K_{p} values for CYP isoform-specific reactions. Each inhibitor was incubated at 37°C for 30 min, in 0.5-ml incubation mixtures containing 0.5 mg/ml microsomes, 50 mM potassium phosphate buffer (pH 7.4), the NADPH-generating system, and 5 µM [14C]ebastine. The assay procedure was essentially the same as that described in Correlation Analyses.

Immunoelimination Studies. Polyclonal anti-human CYP1A1/2 (rabbit serum), CYP2C8/9/19 (goat serum), CYP2D6 (rabbit serum), and CYP3A4/5 (rabbit serum) antibodies raised against purified rat CYP1A2, rat CYP2C13, cDNA-expressed human CYP2D6, and rat CYP3A2, respectively, were purchased from Daichi Pure Chemicals Co. (Tokyo, Japan). Polyclonal anti-human CYP2E1 antibodies (rabbit serum) raised against purified rat CYP2E1 were obtained from Gentest. Monoclonal anti-human CYP2A6 and CYP3A4/5 antibodies (mouse ascites) raised against purified human CYP2A6 and human CYP3A4, respectively, were purchased from Gentest. Immunoelimination studies were carried out by preincubation of antibodies, the corresponding preimmunized antibodies (for polyclonal antibodies), or 25 nm Tris buffer (pH 7.4) (for monoclonal antibodies) with human liver microsomes (41–66 pmol total CYP/ml) or lymphoblastoid microsomes containing cDNA-expressed CYP3A4 (56 pmol CYP3A4/ml), at room temperature for 15 min, before the reaction was started by the addition of ebastine and the NADPH-generating system. The kinetics of formation of M-OH by human liver microsomes in the absence or presence of anti-CYP3A antibodies (1 mg IgG/ml) were examined at liver microsomal protein concentrations of 0.1 mg/ml and ebastine concent-
tions of 0–30 μM. The assay procedure was essentially the same as that described in Correlation Analyses.

Data Analysis. The values were given as means ± SE. Determination of kinetic parameters (Vmax and Km) was accomplished with Enzfitter (Biosoft; Elsevier, Milltown, NJ) curve-fitting software. Correlations of ebastine metabolism with CYP activities were evaluated by least-squares regression analysis.

Results

Ebastine Metabolism by Human Liver Microsomes. In the presence of NADPH, ebastine was metabolized by human liver microsomes to two major metabolites (des-BP and M-OH) (fig. 1), which accounted for >90% of the disappearance of the parent drug; the reactions yielding des-BP and M-OH involved the oxidative dealkylation of the piperidine moiety and the hydroxylation of a methyl group of the tert-butyl substituent, respectively. The rates of formation of both metabolites were proportional to incubation times up to 60 min and protein concentrations up to 1.0 mg/ml at 30 min.

The formation of both metabolites followed Michaelis-Menten kinetics with 2–30 μM ebastine. The kinetic values (mean ± SE) from the four human microsomal preparations were as follows: Kmax for formation of des-BP and M-OH, 5.67 ± 0.64 and 6.55 ± 1.24 μM; Vmax for formation of des-BP and M-OH, 0.613 ± 0.180 and 0.211 ± 0.047 nmol/min/mg protein, respectively. The rate of dealkylation was thus faster than that of hydroxylation, and the dealkylation/hydroxylation rate ratio was 2.9:1, ranging from 1.9:1 to 3.4:1.

Correlation Analyses. Table 1 shows the correlations of the rates of des-BP and M-OH formation with isomform-specific activities in microsomes from 14 human liver samples. In addition, plots of the rates of dealkylation and hydroxylation of ebastine (at ebastine concentrations of 5 and 20 μM) vs. the corresponding rates of testosterone 6β-hydroxylation are shown in fig. 2. For the panel of 14 human liver microsomal preparations, the rates of dealkylation at the two ebastine concentrations (5 and 20 μM) showed statistically significant correlations with CYP3A-mediated testosterone 6β-hydroxylation (Waxman et al., 1988) but not with seven other CYP isomform activities. The results indicate CYP3A participation in the formation of des-BP.

On the other hand, the coefficients for the correlation between ebastine hydroxylation and testosterone 6β-hydroxylation were smaller than those for dealkylation at both concentrations. Furthermore, ebastine hydroxylation was significantly correlated with testosterone 6β-hydroxylation at the high concentration (r = 0.72, p < 0.005) but was not significantly correlated at the low concentration (r = 0.49, p < 0.1). Interestingly, there was no clear correlation between the two pathways for ebastine itself (dealkylation and hydroxylation) at either concentration.

Ebastine Metabolism by cDNA-Expressed Human CYP Enzymes. cDNA-expressed human CYP3A4 alone metabolized ebastine to its metabolites, whereas other isozymes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP4A11) did not (fig. 3). The metabolites formed by CYP3A4 were exclusively two (des-BP and M-OH) under these conditions. The kinetic values for dealkylation and hydroxylation were as follows: Kmax, 11.5 ± 1.5 and 10.0 ± 2.9 μM; Vmax, 0.284 ± 0.015 and 0.023 ± 0.003 nmol/min/nmol of CYP3A4, respectively. The ratio of the rate of dealkylation to that of hydroxylation in this system was 12:1, which was obviously different from the ratio of 3:1 obtained with human liver microsomes (fig. 4). This difference between human liver microsomes and cDNA-expressed CYP3A4 is discussed below.

Chemical Inhibition Studies. Chemical inhibitors were then tested for their effects on ebastine metabolism by the human liver microsomes. The effects of CYP isomform-selective inhibitors on ebastine metabolism are illustrated in fig. 5. CYP inhibitors/substrates such as furafylline (CYP1A2) (Sesardic et al., 1990; Kunze and Trager, 1993), coumarin (CYP2A6) (Yun et al., 1991; Nunoya et al., 1996), orphenadrine (CYP2B6) (Heyn et al., 1996; Reidy et al., 1989), sulfaphenazole (CYP2C2) (Back et al., 1988; Baldwin et al., 1995; Newton et al., 1995), tranylcypromine (CYP2C19) (Inaba et al., 1985), quinidine (CYP2D6) (Newton et al., 1995; Inaba et al., 1985), diethylthiocarbamate (CYP2E1) (Newton et al., 1995; Guengerich et al., 1991; Chang et al., 1994), and lauric acid (CYP4A) (Kawashima et al., 1994) had no inhibitory effects on metabolism. Teconazole (Back et al., 1988; Baldwin et al., 1995; Newton et al., 1995; Maurice et al., 1992) and TAO (Newton et al., 1995; Chang et al., 1994; Pessayre et al., 1983), well-known CYP3A inhibitors, inhibited dealkylation by 100% and 56% and hydroxylation by 57% and 20%, respectively. Furthermore, the CYP3A substrates cyclosporin A (Kronbach et al., 1988) and midazolam (Kronbach et al., 1989) inhibited the reactions but hydroxylation was more resistant to the inhibitors, that is, inhibitory concentrations for dealkylation were >10-fold lower than those for hydroxylation (fig. 6).

Immunoinhibition Studies. Anti-CYP1A1/2,-CYP1A2,-CYP2D6,-CYP2A6, and -2A6 antibodies and two anti-CYP3A4/5 antibodies, raised against purified rat or human CYP isozymes, were tested for their effects on dealkylation and hydroxylation. Anti-CYP3A4/5 antibodies markedly inhibited dealkylation, with little effect on hydroxylation.
Negligible inhibition by other antibodies was seen for the reactions. Fig. 8 shows the effects of anti-CYP3A antibodies on ebastine dealkylation and hydroxylation by cDNA-expressed CYP3A4. Anti-CYP3A antibodies strongly inhibited both dealkylation and hydroxylation.

The kinetics of M-OH formation were then tested in the human microsomal system, under conditions where anti-CYP3A antibodies completely inhibited CYP3A activity for M-6 formation. In contrast to dealkylation, hydroxylation was not influenced by the antibodies, as revealed by insignificant changes in the kinetic parameters; a $K_{m}$ of 4.98 μM and a $V_{max}$ of 0.162 nmol/min/mg protein were obtained in the presence of the antibodies, and a $K_{m}$ of 5.33 μM and a $V_{max}$ of 0.167 nmol/min/mg protein were obtained in the presence of preimmune serum.

**Discussion**

As the first step in the metabolism of ebastine, conversion to two major metabolites, des-BP and M-OH, was demonstrated with human liver microsomes (fig. 1). Des-BP, the N-dealkylation product, is pharmacologically inactive and is excreted after undergoing further metabolism. M-OH undergoes further oxidation to form carebastine, the important active compound possessing potent H1 receptor antagonistic activity. The unchanged form of ebastine in plasma was almost below the detection limit after administration of clinical doses (Yamaguchi et al., 1994); however, it was detected when the drug was coadministered with ketoconazole or erythromycin, both well-known inhibitors of CYP3A (Wiseman and Faulds, 1996). Although this
The formation of des-BP was strongly inhibited by anti-CYP3A4/5 antibodies (fig. 7). Finally, anti-CYP3A4/5 antibodies insignificantly inhibited hydroxylation of ebastine in human liver microsomes, whereas they completely inhibited the hydroxylation catalyzed by cDNA-expressed CYP3A4 (figs. 7 and 8). In addition, the kinetics of formation of des-BP in human liver microsomes were not at all influenced by the varying amounts of antibodies, under conditions in which the formation of des-BP was completely inhibited. Regarding the third and fourth points, effects of chemical inhibitors on hydroxylation were noted at very high concentrations (100 µM), whereas anti-CYP3A4 antibodies showed no effect on hydroxylation. The difference might be explained by the loss of specificity of chemical inhibitors at extremely high concentrations, which is a well-known phenomenon with ketoconazole (Back et al., 1988; Baldwin et al., 1995; Newton et al., 1995; Maurice et al., 1992).

Furthermore, the correlation analyses showed that the formation of M-OH was significantly correlated with testosterone 6β-hydroxylation only at the high concentration (20 µM) of ebastine and was not significantly correlated at the low concentration (5 µM), with the lower level being clinically relevant. These facts suggest that the contribution of CYP3A to ebastine hydroxylation is dependent on the ebastine concentrations. Because tissue concentrations of unbound drugs are generally much lower than their Km values, the considerations discussed above strongly suggest that an unidentified enzyme, other than CYP3A, could play an important role in vivo. According to Shimada et al. (1994), the cDNA-expressed CYP isoforms used in this study could account for up to 70% of the total CYP enzyme content in the liver. In conclusion, isoforms other than CYP3A may also be involved in the hydroxylation of ebastine in humans.

Terfenadine, a prototype for nonsedative antihistamine agents, has two similar major metabolic pathways, i.e. dealkylation and hydroxylation (Kivisto et al., 1994; Jurima-Romet et al., 1989). However, unlike for ebastine, both hydroxylation and dealkylation are catalyzed predominantly by CYP3A4 (Jurima-Romet et al., 1989; Yun et al., 1993; Ling et al., 1995; Rodrigues et al., 1995), as clearly shown by the findings that the dealkylation/hydroxylation rate ratio for cDNA-expressed human CYP3A4 (1:1.2) was almost the same as that for human liver microsomes (Yun et al., 1993), that in the correlation analyses dealkylation and hydroxylation of terfenadine were highly correlated (r = 0.90, p < 0.0004) (Ling et al., 1995), and that the hydroxylation pathway of terfenadine was strongly inhibited by 5 µM ketoconazole (>80%) (Ling et al., 1995; Rodrigues et al., 1995) and by anti-CYP3A antibodies (>90%) (Yun et al., 1993). The difference in the enzymes responsible for ebastine and terfenadine metabolism would influence the in vivo pharmacokinetic behavior of the two drugs when a CYP3A inhibitor is coadministered. Enzymatic mechanisms for the drug-drug interactions between ebastine or terfenadine and a CYP3A inhibitor would differ.

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