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Identification of Human Liver Cytochrome P450 Enzymes that Metabolize the Nonsedating Antihistamine Loratadine

FORMATION OF DESCARBOETHOXYLORATADINE BY CYP3A4 AND CYP2D6

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ABSTRACT. [^{3}H]Loratadine was incubated with human liver microsomes to determine which cytochrome P450 (CYP) enzymes are responsible for its oxidative metabolism. Specific enzymes were identified by correlation analysis, by inhibition studies (chemical and immunoinhibition), and by incubation with various cDNA-expressed human P450 enzymes. Descarboethoxyloratadine (DCL) was the major metabolite of loratadine detected following incubation with pooled human liver microsomes. Although DCL can theoretically form by hydrolysis, the conversion of loratadine to DCL by human liver microsomes was not inhibited by the esterase inhibitor phenylmethylsulfonyl fluoride (PMSF), and was dependent on NADPH. A high correlation ($r^2 = 0.96$, N = 10) was noted between the rate of formation of DCL and testosterone 6β-hydroxylation, a CYP3A-mediated reaction. With the addition of ketoconazole (CYP3A4 inhibitor) to the incubation mixtures, the residual rate of formation of DCL correlated ($r^2 = 0.81$) with that for dextromethorphan O-demethylation, a CYP2D6 reaction. Rabbit polyclonal antibodies raised against the rat CYP3A1 enzyme (5 mg IgG/nmol P450) and troleandomycin (0.5 μM), a specific inhibitor of CYP3A4, decreased the formation of DCL by 53 and 75%, respectively, when added to 1.42 μM loratadine microsomal incubations. Quinidine (5 μM), a CYP2D6 inhibitor, inhibited the formation of DCL approximately 20% when added to microsomal incubations of loratadine at concentrations of 7–35 μM. Incubation of loratadine with cDNA-expressed CYP3A4 and CYP2D6 microsomes catalyzed the formation of DCL with formation rates of 135 and 633 pmol/min/nmol P450, respectively. The results indicated that loratadine was metabolized to DCL primarily by the CYP3A4 and CYP2D6 enzymes in human liver microsomes. In the presence of a CYP3A4 inhibitor, loratadine was metabolized to DCL by the CYP2D6 enzyme. Conformational and electrostatic analysis of loratadine indicated that its structure is consistent with substrate models for the CYP2D6 enzyme. BIOCHEM PHARMACOL 51;2:165–172, 1996.

KEY WORDS. cytochrome P450; *in vitro* metabolism; liver; loratadine; human; molecular modeling

Loratadine [ethyl-4-(8-chloro-5,6-dihydro-11*H*-benzo[5,6]-cyclohepta[1,2-*b*]pyridin-11-ylidene)-1-piperidinecarboxylate Fig. 1] is a once-a-day nonsedating antihistamine indicated for the relief of nasal and non-nasal symptoms of seasonal allergic rhinitis [1, 2]. It is currently marketed in some 88 countries, including the United States (as CLARITIN®). Studies on the metabolism of loratadine in laboratory animals have shown that the drug is well absorbed, but undergoes extensive first-pass metabolism [3]. Only trace amounts of parent drug are detected in the urine or bile of rats over the 24-hr period following a single oral or intravenous dose.‖ From the metabolites identified by mass spectrometry and NMR in human, monkey, and rat urine following oral dosing with [^{14}C]loratadine, it was proposed that loratadine is initially hydrolyzed to form DCL¶ (Fig. 1), a metabolite with antihistaminic properties [4], which then undergoes further oxidative metabolism to generate hydroxylated metabolites of DCL.‖ In humans, high plasma concentrations of the active metabolite DCL have been found following dosing with loratadine [5]. Since CYP hemoproteins play a major role in the oxidative biotransformation of xenobiotics, an evaluation was made of the specific CYPs involved in the metabolism of loratadine. The objective of this study was to determine which CYP enzymes catalyze the Phase I oxidative biotransformation of loratadine. The experimental methods used included correlation analysis [6], chemical inhibition studies, immunoinhibition studies, metabolism studies with cDNA-expressed human CYP microsomes, and molecular modeling.

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¶ *Abbreviations:* DCL, descarboethoxyloratadine; CYP, cytochrome P450; TAO, troleandomycin; and PMSF, phenylmethylsulfonyl fluoride.

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‖ Nicola Zampaglione, unpublished results. Cited with permission.

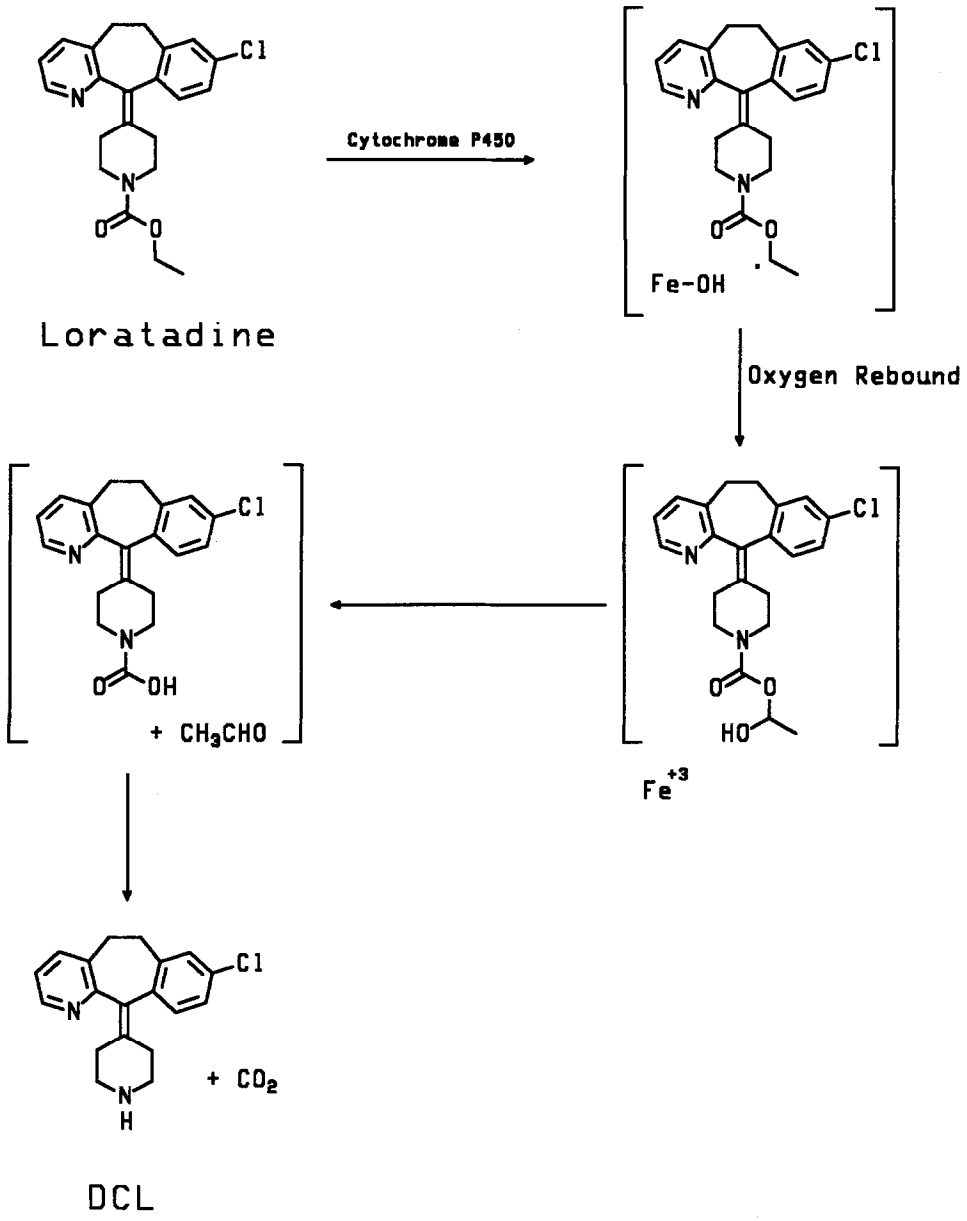

FIG. 1. Cytochrome P450 mechanism for DCL formation from loratadine (bracketed structures have not been isolated).

MATERIALS AND METHODS

Chemicals

[^{3}H]Loratadine, uniformly labeled at positions 2 and 6 of the piperidine ring, was purchased from Amersham (Arlington Heights, IL). The specific activity was 350 mCi/mmol, and the radiochemical purity was at least 97.7% by radiochemical HPLC detection at the time detection at the time the study was initiated. DCL, SCH 37370, 1-acetyl-4-(8-chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-ylidene) piperidine (HPLC internal standard), and ketoconazole were obtained from Schering-Plough Research Institute, Kenilworth, NJ. Glucose-6-phosphate dehydrogenase and glucose-6-phosphate were purchased from Calbiochem (San Diego, CA). NADPH and TAO were purchased from the Sigma Chemical Co. (St. Louis, MO). Quinidine and PMSF were purchased from the Aldrich Chemical Co. (Milwaukee, WI).

Enzymes and Antibodies

Individual and pooled human liver microsomes were obtained from the HepatoScreen™ test kit, available commercially from Human Biologics Inc. (Phoenix, AZ). Rabbit polyclonal antibody against rat CYP3A1 was also purchased from Human Biologics. cDNA-expressed human CYP microsomes were purchased from Gentest (Woburn, MA).

Inhibition Assays

Solutions containing 1–5% [^{3}H]loratadine (v/v) and pooled human liver microsomes were prepared by initially adding aliquots of the [^{3}H]loratadine stock solution to separate tubes. The solvent was removed by evaporation under vacuum using a Savant Instruments model SC 200 Speed-Vac® (Farmingdale, NY). A standard volume of ethanol (10 μL) was re-

added to each tube to ensure adequate solubility of loratadine in the aqueous buffer solutions. An aliquot of diluted pooled human liver microsomes was added to the tubes containing [^{3}H]loratadine for a microsomal concentration of 1 nmol CYP/mL.

Aliquots of the loratadine and microsomal solution were added to preincubated (3 min) open air tubes containing 1 mM NADPH, 0.1 mM $MgCl_2$, 1 U glucose-6-phosphate dehydrogenase/mL, 5 mM glucose-6-phosphate, and ketoconazole or quinidine in potassium phosphate buffer, pH 7.4, at 37°. A total of 0.05 nmol of CYP was used in each incubation. The concentrations used in these experiments ranged from 7 to 35 μM for loratadine, 0.1 to 5 μM for ketoconazole, and 5 to 250 μM for quinidine. Inhibitors were added to the NADPH-generating system in potassium phosphate buffer, pH 7.4, ethanol (≤1%) or DMSO (≤1%) for a final total volume of 0.2 mL. In the TAO experiments, the inhibitor (concentration range 0.5 to 10 μM) was incubated for 30 min with pooled human liver microsomes (0.05 nmol CYP/incubation) and the NADPH-generating system described above in open air tubes in a total volume of 0.1 mL. Following preincubation, a solution of [^{3}H]loratadine and additional NADPH cofactor in potassium phosphate buffer were added to yield a final loratadine concentration of 1.42 μM in a total volume of 1.0 mL.

Immunoinhibition experiments with loratadine were performed in glass tubes containing aliquots of either control IgG or the rabbit polyclonal antibody raised against rat CYP3A1, the NADPH-generating system described previously, pooled human liver microsomes (final incubation concentration of 0.05 nmol CYP/mL), and sufficient potassium phosphate buffer, pH 7.4, for a final incubation volume of 1 mL. Following preincubation of the mixture for 3 min at 37°, loratadine was added to each tube (1.42 μM concentration), and the mixture was incubated for another 10 min. All incubations were conducted in duplicate and those without NADPH added to the cofactor solution were used as controls. The suspensions were incubated in an Aquatherm® waterbath shaker (New Brunswick Scientific, Edison, NJ) for 10 min, and all incubations were terminated by the addition of 250 μL of acetonitrile.

Correlation Analysis

Correlation analysis was performed with ten human liver microsomal samples provided in the HepatoScreen™ test kit. Biochemical activity data for specific CYP enzymes were provided for each sample in the kit. Test kit data were verified by reassay of the ethoxyresorufin O-deethylation activity in selected human liver samples in the test kit. Aliquots of diluted human liver microsomal samples from the HepatoScreen™ test kit and the NADPH-generating system described above in potassium phosphate buffer, pH 7.4, were incubated for 3 min at 37° in open air tubes (final volume of 1 mL). The protein concentration used in each microsomal incubation was 0.13 mg/mL. Aliquots of a loratadine solution were added (1.42 μM concentration) to each tube, and the mixtures were incubated for 10 min at 37°. Incubations were terminated with the addition of 1 mL of acetonitrile and processed as described below. The rate of formation of DCL was measured in each of the ten human liver microsomal samples provided in the HepatoScreen™ test kit, and the *in vitro* rates of formation were correlated with P450 enzyme activity data provided with the HepatoScreen™ test kit (see Table 1). Since the biochemical activity data provided with the kit are mediated by specific CYP enzymes, high correlation coefficients would indicate that similar enzymes were involved in the formation of DCL.

TABLE 1. Correlation Between Rate of Formation of DCL and Other P-450-Dependent Reactions in Human Liver Microsomes in the Presence and Absence of Ketoconazole

Enzymatic test reaction	P450 involved	DCL correlation coefficient (r^2)*	
			+ 500 nM Ketoconazole
Ethoxyresorufin O-deethylation	CYP 1A2	0.05	0.28
Caffeine N3-demethylation	CYP 1A2	0.11	0.37
Coumarin 7-hydroxylation	CYP 2A6	0.12	0.03
S-Mephenytoin 4′-hydroxylation	CYP 2C19	0.00	0.16
Tolbutamide methyl-hydroxylation	CYP 2C9/10	0.49	0.03
Dextromethorphan O-demethylation	CYP 2D6	0.03	0.81
Chlorzoxazone 6-hydroxylation	CYP 2E1	0.21	0.00
Testosterone 6β-hydroxylation	CYP 3A4/5	0.96	0.01
Lauric acid 11-hydroxylation		0.20	0.00
Lauric acid 12-hydroxylation	CYP 4A9/11	0.35	0.03

* Ten samples.

cDNA-Expressed Human P450 Microsomal Incubations

Solutions containing sample aliquots of cDNA-expressed human CYP 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A4, or control microsomes from Gentest and the NADPH-generating system described above were prepared in open air plastic test tubes with potassium phosphate buffer, pH 7.4. After preincubation for 3 min at 37°, loratadine (final concentration, 1.42 μM) was added, and the incubations were terminated after 20 min by the addition of 1 mL of acetonitrile.

HPLC Analysis

Incubation mixtures were centrifuged for 10 min at 1000 *g*, and the supernatant was removed and transferred to new test tubes. Solutions of DCL and SCH 37370 (internal standard) were added to the samples as HPLC reference standards. The tubes were dried *in vacuo* with a Savant Instruments model SC200 Speed-Vac®. The residue was reconstituted in the HPLC mobile phase, and the samples were analyzed with a Waters (Milford, MA) automated microprocessor-controlled HPLC system consisting of a model 712 WISP autosampler, model 6000A solvent-pumps, and either a model 490E programmable wavelength UV-detector in series with a Radiomatic A-250 radioflow detector (Radiomatic Instruments, Tampa, FL) or a Waters model 480 UV detector in series with a β-Ram® radioflow detector (IN/US Systems Inc., Fairfield, NJ) for tandem detection ($UV_{254\ nm}$ and [^{3}H]radioactivity). Separation of loratadine and metabolites was accomplished on a Waters C-18 Novapak® (10 μm particle size; 3.9 mm × 150 mm) analytical HPLC column with a linear gradient from an initial 65% methanol/36% 0.2 M ammonium acetate, pH 6.0, buffer to a final 15% methanol/85% 0.2 M ammonium acetate, pH 6.0, buffer at a flow rate of 1 mL/min over a 30-min time interval. The retention time of DCL was approximately 18 min. Metabolites were identified by a comparison of HPLC peak retention times to that of authentic reference standards and characterized structurally by LC/MS.

Molecular Modeling

Monte Carlo conformational searches were performed using Macromodel v4.5 [7] with the mm3 force field in order to explore the conformational space of loratadine and dextromethorphan, a substrate for the CYP2D6 enzyme [8]. The search was performed for loratadine from 4000 starting points, but for the more rigid dextromethorphan, 2500 starting points were used. The two lowest energy conformers of loratadine, differing by less than 0.1 kcal/mol, were found 22 and 14 times

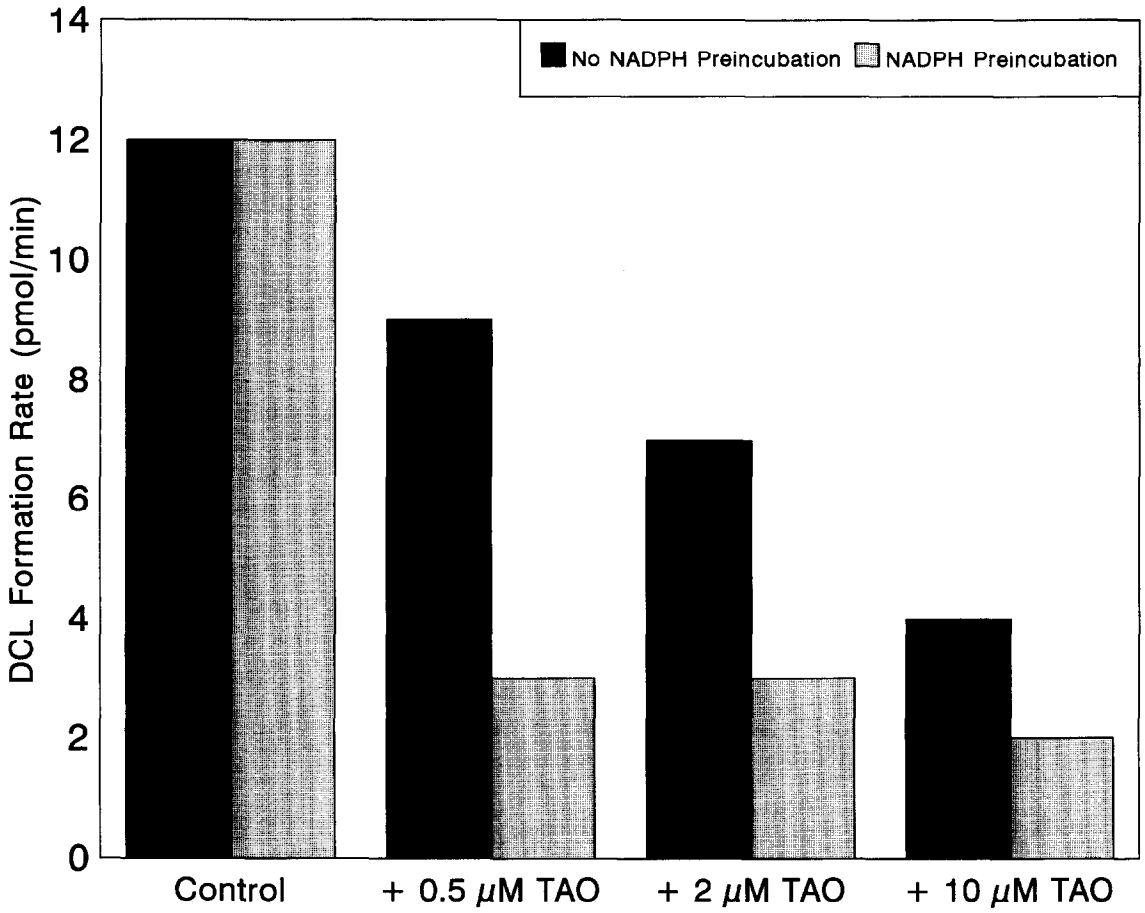

FIG. 2. Effect of troleandomycin, a CYP3A4 inhibitor, on the formation of DCL from loratadine by human liver microsomes. Duplicate samples were analyzed with or without preincubation of troleandomycin with human liver microsomes and NADPH.

during the search (indicating good coverage of conformation space) and had basic nitrogen (pyridinyl nitrogen) to oxidation-site distances of 6.48 and 7.43 Å. For dextromethorphan, the two lowest energy conformers, differing by less than 0.1 kcal/mol, were found 88 and 76 times during the search and had basic nitrogen to oxidation-site distances of 8.04 and 7.55 Å. The 7.43 Å conformation of loratadine and the 7.55 Å conformation of dextromethorphan were taken as model conformations. Molecular electrostatic potentials were calculated using these mm3-minimized starting geometries. Potentials were calculated on the electron density isopotential surfaces at 0.002 e/Å^3 using MNDO with the SPARTAN program [9]. All calculations were performed on Silicon Graphics Challenge XL or Silicon Graphics Indigo2 workstations.

RESULTS AND DISCUSSION

In Vitro *Metabolism of Loratadine*

DCL was the predominant metabolite identified following incubation of [^{3}H]loratadine with pooled human microsomes by HPLC-cochromatography with an authentic standard of DCL and by LC/MS. This finding supports the earlier suggestion that loratadine is initially metabolized *in vivo* to DCL, which then undergoes further oxidative metabolism to generate hydroxylated metabolites of DCL. Studies were conducted to determine whether the mechanism for the hydrolysis of the carbamate ester functionality to form DCL was mediated by CYP. The formation of DCL was found to require NADPH, consistent with a CYP-dependent process. Addition of PMSF, an inhibitor of esterases [10], to the incubation mixture had no effect on the rate of DCL formation. Negligible amounts of DCL were detected in human liver cytosol. These results indicated that the oxidative metabolism of loratadine to DCL is a CYP-dependent reaction. Guengerich *et al.* [11, 12] have reported that the hydrolysis of substituted pyridine dicarboxylic acid esters is catalyzed by CYP. The process is thought to occur by initial hydrogen atom abstraction from the carbon atom of the ester group adjacent to the carboxylate functionality, oxygen rebound from CYP to form a hemiacetal, and collapse of the hemiacetal to generate a carboxylic acid and an aldehyde from the ester group. If a similar process occurs with loratadine, the carbamic acid intermediate formed would be expected to decarboxylate to form DCL (Fig. 1).

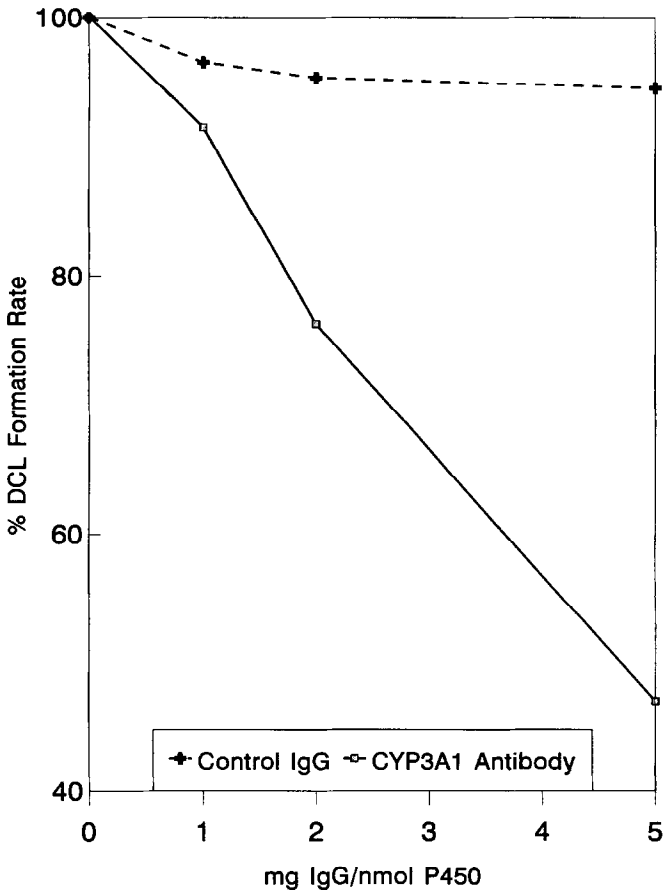

FIG. 3. Effect of rat anti-CYP3A1 on the formation of DCL from loratadine by human liver microsomes. Data were determined from duplicate samples analyzed for each concentration of anti-CYP3A1 and are displayed as percentages of the initial formation rate without antibody.

Correlation Studies

The formation of DCL by ten samples of human liver microsomes (HepatoScreen™ test kit) was monitored because it is a major circulating metabolite in humans [5]. The sample-to-sample variation for DCL formation was highly correlated ($r^2 = 0.96$, with a ≥99.9% confidence limit [13]) with the test kit data for testosterone 6β-hydroxylation, a marker for CYP3A. All other correlations between the Human Biologics assay data and DCL formation were less than $r^2 = 0.50$, suggesting that loratadine is metabolized to DCL primarily by a CYP3A enzyme (Table 1).

Ketoconazole and other antifungals that act by blocking the conversion of lanosterol to ergosterol [14] by inhibiting the CYP-dependent 14α-demethylation of lanosterol have also been shown to inhibit mammalian CYP enzymes. Ketoconazole, for example, inhibits the CYP3A-dependent metabolism of cyclosporin A and cortisol by human liver microsomes [15–17]. Therefore, incubations of loratadine with the HepatoScreen™ test kit human liver microsomes were conducted in the presence of ketoconazole (500 nM) to determine if other CYP enzymes can metabolize loratadine to DCL when CYP3A4 is inhibited. Preliminary studies indicated that a concentration of 500 nM ketoconazole inhibited the conversion of loratadine to DCL by 77% at a loratadine substrate concentration of 7.1 μM; however, inhibition of the formation of DCL by ketoconazole appeared to plateau at higher concentrations. In the presence of 500 nM ketoconazole, a high correlation ($r^2 = 0.81$) between the residual rate of formation of DCL and the HepatoScreen™ test kit data for dextrometh-orphan O-demethylation, a CYP2D6-mediated reaction, was noted (Table 1). All other correlations with the HepatoScreen™ test kit data were less than $r^2 = 0.40$. These findings indicated that loratadine was metabolized to

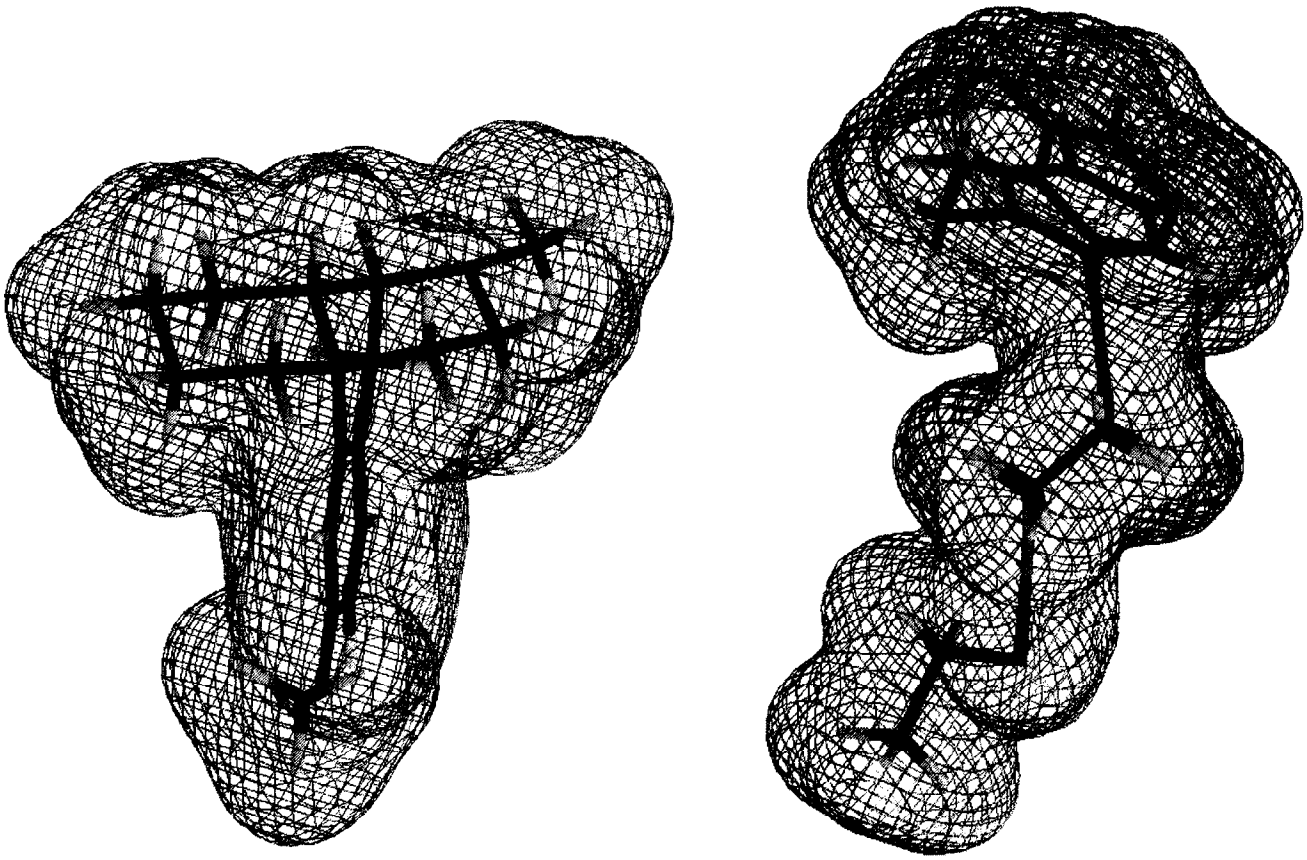

FIG. 4. (Left panel) Molecular electrostatic potential of dextromethorphan. Regions of negative potential are indicated in red. (Right panel) Molecular electrostatic potential of loratadine. Regions of negative potential are indicated in red.

DCL, in the presence of a CYP3A4 inhibitor, primarily by CYP2D6.

Inhibition Studies

The formation of DCL from loratadine was inhibited when ketoconazole was included at concentrations of 0.1, 0.5, and 5 μM in the incubation mixtures containing pooled human liver microsomes. The inhibition ranged from 54 to 89% at loratadine concentrations ranging from 7 to 35 μM. TAO, a CYP3A4 inhibitor [18, 19], also inhibited the formation of DCL from loratadine, especially when preincubated with microsomes and NADPH (Fig. 2). TAO, at a concentration of 0.5 μM, inhibited the rate of DCL formation by 75% at a loratadine concentration of 1.42 μM following a 30-min preincubation with NADPH and microsomes. Quinidine, a CYP2D6 inhibitor [20], at a concentration of 5 μM, produced less than 20% inhibition in the formation of DCL from loratadine at concentrations of 7–35 μM. The results of these microsomal incubations of loratadine with specific CYP chemical inhibitors are in agreement with the initial findings using the HepatoScreen™ test kit. Addition of ketoconazole and quinidine, the chemical inhibitors of the CYP3A4 and CYP2D6 enzymes, to human liver microsomal incubation mixtures containing loratadine decreased the amount of DCL formed.

The rabbit polyclonal antibody against rat CYP3A1 is known to recognize and cross-react with enzymes in the human CYP3A subfamily [21, 22]. Addition of this rabbit polyclonal antibody to loratadine microsomal incubation mixtures inhibited the formation of DCL (Fig. 3) in a concentration-dependent manner. The formation of DCL was inhibited by 48% at a concentration of 5 mg CYP3A1 antibody/nmol P450 in the incubation mixture, compared with loratadine incubations with control IgG.

cDNA-Expressed Human P450 Microsomal Incubations

Incubations of loratadine with various cDNA-expressed human P450 microsomes were used to confirm the correlation and inhibition results. DCL was detected by HPLC when loratadine was incubated with cDNA-expressed CYP3A4 and CYP2D6 microsomes in the presence of NADPH. The catalytic rates of formation using the CYP3A4 and CYP2D6 microsomes were 135 and 633 pmol/min/nmol P450, respectively. While these results further confirm the correlation and inhibition studies, they do not address the relative contribution of both enzymes in the formation of DCL with human

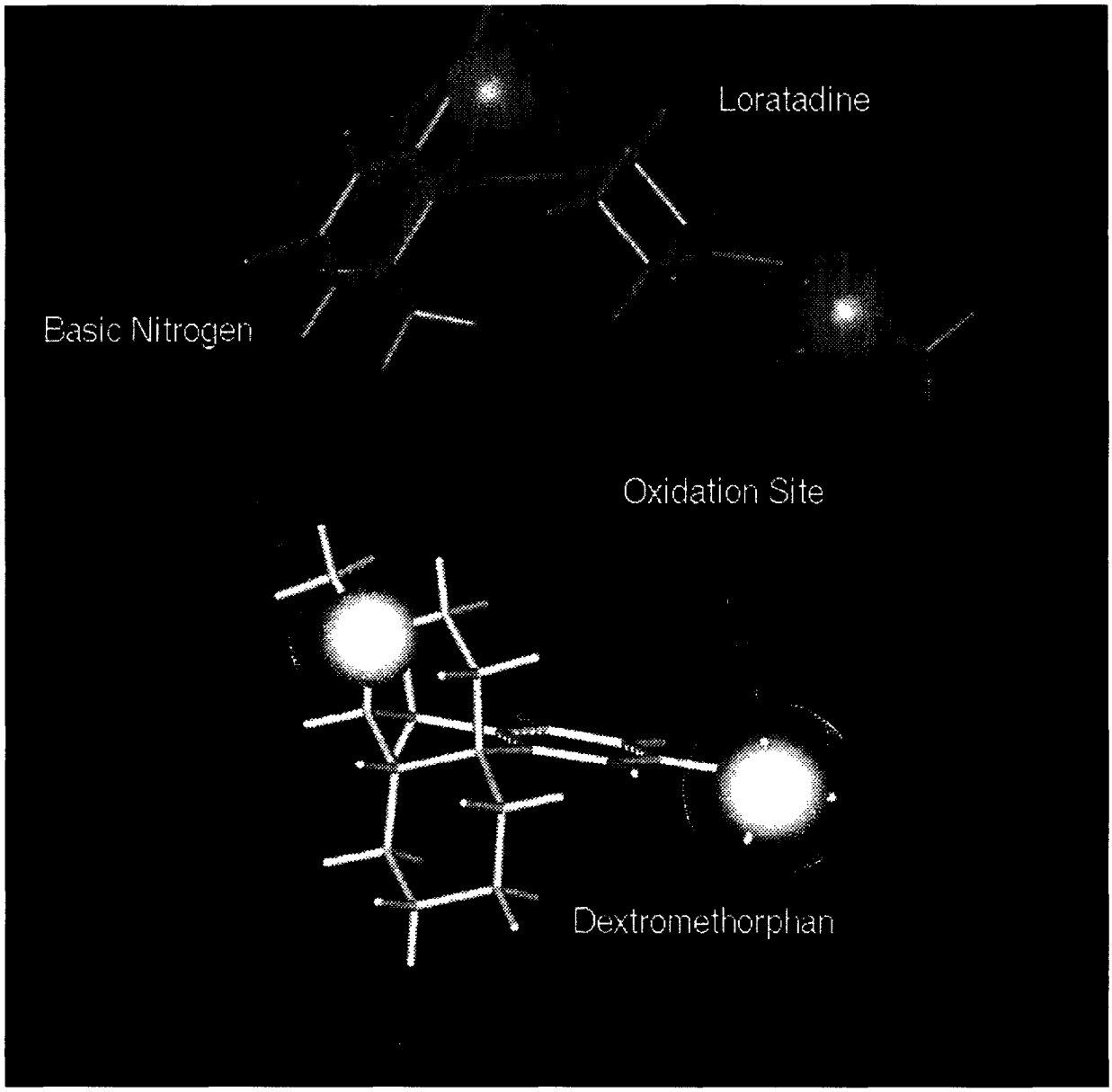

FIG. 5. Model conformations of loratadine and dextromethorphan. The basic nitrogen to oxidation-site distance is 7.43 Å in loratadine and 7.55 Å in dextromethorphan.

liver microsomes.* CYP3A4 catalytic activities have been shown to be variable in vector-based systems [23]. No radioactive peak coeluting with the DCL standard was detectable when loratadine was incubated with other cDNA-expressed CYP microsomes.

Computational Analysis

A review of compounds known to be metabolized by the CYP2D6 enzyme have revealed common structural features, notably that all possess one or more basic nitrogen atoms [24, 25]. Wolff *et al.* [24] proposed a model in which a number of compounds, including debrisoquine, could be superimposed with the oxidation site about 5 Å from a basic nitrogen. Meyer *et al.* [25] have proposed a model in which this distance is about 7 Å. Neither model can accommodate all the compounds known to be metabolized by the CYP2D6 enzyme. Koymans *et al.* [26] have proposed a "bidentate carboxylate hypothesis," in which they divide the substrates into "5-Å substrates," e.g. debrisoquine, and "7-Å substrates," e.g. dextromethorphan, and propose that the basic nitrogen interacts with one or the other of the carboxylic oxygens of a carboxylate group on the protein. Koymans *et al.* demonstrate that both the 5-Å and the 7-Å substrates can fit this model, and can be aligned such that there is a planar region near the

* Parkinson A, *In vitro* approaches to studying human P450 enzymes. In: *Sixth North American ISSX Meeting, Raleigh, NC, 23–27 October 1994. ISSX Proceedings* **6:** 5, 1994.

oxidation site, and a region of negative electrostatic potential (due to the presence of either an N-atom, an O-atom, or a pi-system) above the planar part of the molecule in proximity to the oxidation site [26].

Conformational and electrostatic analysis of loratadine indicates that the structure fits this model. Specifically, the distance between the oxidation site and the pyridinyl nitrogen (basic nitrogen) in the two lowest energy conformers of loratadine is 6.48 and 7.43 Å. The NCOO-plane in the 7.43 Å-structure is in the position and orientation consistent with the model, and in a region of negative molecular electrostatic potential (Fig. 4). Thus, loratadine fits the model of Koymans *et al.* [26] and acts as a 7 Å (dextromethorphan-like) substrate (Fig. 5).

In conclusion, DCL was shown to be the major metabolite identified by HPLC co-chromatography with an authentic reference standard and by LC/MS analysis following incubation of loratadine with pooled human liver microsomes. Formation of DCL by human liver microsomes was shown to be CYP dependent. Results of correlation analysis and inhibition studies indicated that the human CYP enzymes primarily responsible for the formation of DCL were CYP3A4 and CYP2D6. These results were confirmed further by incubations of loratadine with cDNA-expressed human P450 enzymes. Only cDNA-expressed CYP2D6 and CYP3A4 microsomes metabolized loratadine to DCL. Molecular modeling indicated that the structure and electrostatic properties of loratadine are consistent with published substrate models [26] of the CYP2D6 enzyme. From these studies, it would appear that the human CYP3A4 and CYP2D6 enzymes are the predominant enzymes involved in the oxidative metabolism of loratadine to DCL.

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