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Binding Characteristics of Cetirizine and Levocetirizine to Human H_1 Histamine Receptors: Contribution of Lys^{191} and Thr^{194}

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ABSTRACT

Competition experiments with [^{3}H]mepyramine showed that cetirizine and its enantiomers, levocetirizine and (*S*)-cetirizine, bound with high affinity and stereoselectivity to human H_1 histamine receptors (K_i values of 6, 3, and 100 nM, respectively). Cetirizine and levocetirizine were 600-fold more selective for H_1 receptors compared with a panel of receptors and channels. Binding results indicated that the interaction between cetirizine, its enantiomers, and histamine is compatible with a competitive behavior, in contrast with the noncompetitive profile of cetirizine and levocetirizine observed in isolated organs. Binding kinetics provided a suitable explanation for this observation, because levocetirizine dissociated from H_1 receptors with a half-time of 142 min; that of (*S*)-cetirizine was only 6 min, implying that the former could act as a pseudo-irreversible antagonist in functional studies. The carboxylic function of levocetirizine seemed responsible for its long dissociation time. Indeed, hydroxyl or methyl ester analogs dissociated more rapidly from H_1 receptors, with half-times of 31 min and 7 min, respectively. The importance of the carboxylic function of levocetirizine for the interaction with the H_1 receptor was further supported by the results from the mutation of Lys^{191} to Ala^{191}. This mutation decreased the dissociation half-time of levocetirizine from 142 to 13 min and reduced its affinity from 3 to 12 nM, whereas the affinity and dissociation kinetics of hydroxyl and methyl ester analogs were hardly affected. The mutation of Thr^{194} reduced the binding stereoselectivity by selectively enhancing the affinity of the distomer.

The bioamine histamine produces a variety of physiological and pathophysiological effects through binding and activation of histamine receptors belonging to the superfamily of seven transmembrane G-protein-coupled receptors (Hill et al., 1997). Today, four human histamine receptor subtypes have been cloned: H_1 (Moguilevsky et al., 1994), H_2 (Gantz et al., 1991), H_3 (Lovenberg et al., 1999), and, more recently, H_4 (Oda et al., 2000). H_1 receptors induce smooth muscle contraction and increase vascular permeability and H_1 antagonists constitute a medication of choice to alleviate the symptoms of allergies.

Cetirizine and levocetirizine are second-generation antihistamines. As opposed to first generation drugs, exemplified by hydroxyzine, chlorpheniramine, diphenhydramine, or ketotifen, second-generation drugs are nonsedating or less sedating, probably because of an improved H_1 binding selectivity and reduced brain penetration (Timmerman, 1999). Structure-activity relationships and site-directed mutagenesis experiments performed with the guinea pig H_1 receptor have provided data that have led to model pharmacophores of H_1 antagonists (ter Laak et al., 1995; Wieland et al., 1999). Since the cloning of the H_1 receptor, several studies have been published on mutant receptors designed to better identify the binding pocket and the amino acids residues involved in the binding of histamine and histamine antagonists (Fig. 1). This is of particular interest today in light of recent findings showing that most histamine H_1 antagonists exhibit inverse agonist properties (Bakker et al., 2000). Asp^{107}, located in the third transmembrane domain of the human receptor, is crucial for the affinity of histamine and histamine antagonists (Ohta et al., 1994); this amino acid is a hallmark of G-

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ABBREVIATIONS: [^{3}H]RX821002, 1,4-[6,7(*n*)-^{3}H]benzodiazoxan-2-methoxy-2-yl)-2-imidazoline hydrochloride; [^{3}H]mepyramine, pyridinyl-5-[^{3}H]pyrilamine; SCH23390 *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SPA, scintillation proximity assay; α-MEM, α-Modified Eagle's minimal essential medium; CHO, Chinese hamster ovary; [^{3}H]NMS, *l*-*N*-methyl-[^{3}H]scopolamine methyl chloride; [^{3}H]N^α-methylhistamine, *N*-α-[methyl-^{3}H]methylhistamine; [^{3}H]8-OH-DPAT, [propyl-2,3-ring-1,2,3-^{3}H]8-hydroxy-dipropylaminotetralin; [^{3}H]CGP-12177, [5,7-^{3}H](−)CGP-12177; [^{3}H]spiperone, [benzene ring-^{3}H]spiperone; [^{3}H]DPCPX, 8-[dipropyl-2,3-^{3}H(N)]cyclopenthyl-1,3-dipropylxanthine; [^{3}H]ketanserin, [ethylene-^{3}H]ketanserin hydrochloride; [^{3}H]tiotidine, [methyl-^{3}H]tiotidine; [^{3}H]prazosin, [7-methoxy-^{3}H]prazosin.

protein-coupled receptors, whose natural ligands are bioamines, and is responsible for forming an ionic bond with the protonated nitrogen of the neurotransmitter (Hibert et al., 1991). Asn^{198} (Leurs et al., 1994; Ohta et al., 1994; Moguilevsky et al., 1995) and Lys^{200} (Leurs et al., 1995) are also involved in histamine binding to the H_1 receptor in human and guinea pig, respectively. By analogy with the histamine H_2 receptor, Thr^{194} was expected to participate in the binding of histamine to human H_1 receptors, but the mutation of this residue into Ala led to a receptor that kept its ability to bind histamine and histamine antagonists with affinities very similar to that of the wild-type receptor (Leurs et al., 1994; Ohta et al., 1994). However, we have shown that the mutation of Thr^{194} to Ala decreased the stereoselectivity of the enantiomers of cetirizine by increasing the affinity of the distomer (Moguilevsky et al., 1995). Finally, Lys^{200}, the guinea pig equivalent of human Lys^{191}, was reported to interact with the carboxylic acid moiety of two second-generation antagonists, acrivastine and cetirizine (Wieland et al., 1999). In this report, we further explore the binding characteristics of cetirizine and its enantiomers to the wild-type human H_1 receptor in comparison with receptors bearing mutations at key amino acids (Lys^{191} and Thr^{194}) known to be involved in ligand binding. Close structural analogs of cetirizine were also included in this study to explore the role of the carboxyl group in binding to the H_1 receptor, under both equilibrium and nonequilibrium conditions.

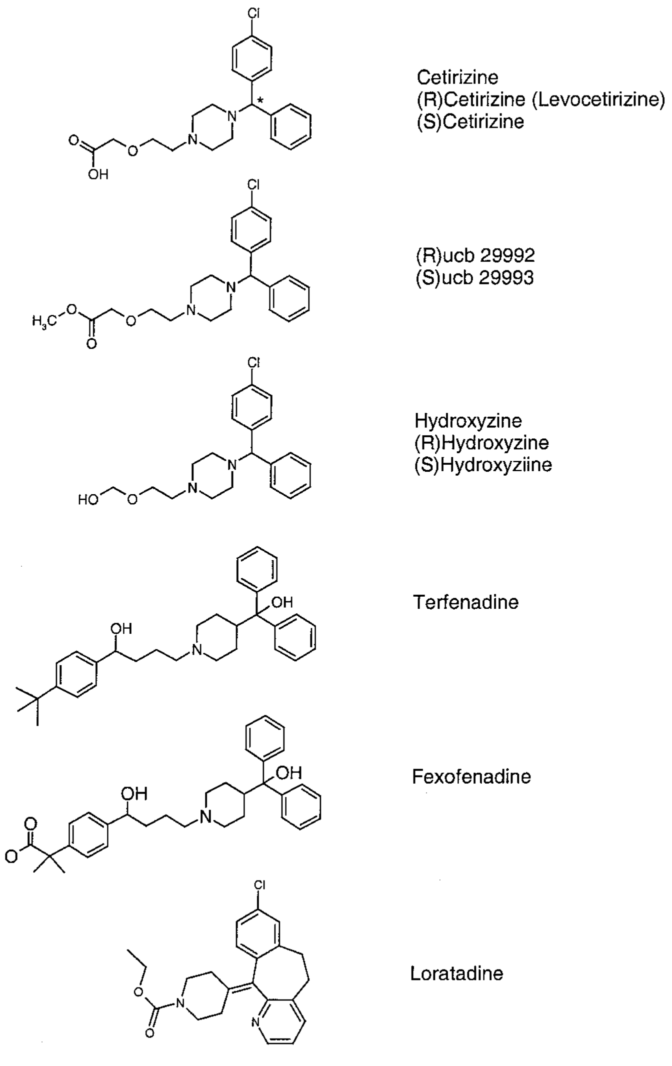

Fig. 1. Chemical structures of H_1 antagonists.

Materials and Methods

Chemicals.

Cetirizine (Zyrtec; UCB Group, Brussels, Belgium), hydroxyzine, and their respective enantiomers [levocetirizine (Xyzal; UCB Group), (*S*)-cetirizine, (*S*)-hydroxyzine, and (*R*)-hydroxyzine (all as dihydrochloride salts)], (*R*)-ucb 29992, and (*S*)-ucb 29993 (as dimaleates) were synthesized at UCB SA Pharma Sector (Braine l'Alleud, Belgium). Fexofenadine was purchased from Ultrafine Chemicals (Manchester, UK). Histamine, (+)-chlorpheniramine, terfenadine, atropine, 2-chloroadenosine, chlorpromazine, ranitidine, pirenzepine, pargyline, and adenosine deaminase (EC 3.5.4.4. from bovine spleen) were from Sigma-Aldrich (Bornem, Belgium). WB-4101, (±)-isoproterenol, thioperamide, ritanserin, ketanserin, buspirone, (±)-propranolol, phentolamine, RX821002, R^{α}-methylhistamine, and butaclamol were purchased from Sigma/RBI (Natick, MA). Serotonin was purchased from Fluka (Bornem, Belgium). Pyridinyl-5-[^{3}H]pyrilamine (27 Ci/mmol), *l*-*N*-methyl-[^{3}H]scopolamine methyl chloride (83 Ci/mmol), [^{3}H]RX821002 (59 Ci/mmol), [^{3}H]SCH23390 (80 Ci/mmol) and wheat germ agglutinin-coated polyvinyltoluene SPA beads were purchased from Amersham Biosciences (Rosendaal, the Netherlands). *N*-α-[methyl-^{3}H]methylhistamine (79 Ci/mmol), [propyl-2,3-ring-1,2,3-^{3}H] 8-hydroxy-2-dipropylaminotetralin (154 Ci/mmol), [5,7-^{3}H](−)CGP-12177 (45 Ci/mmol), [benzene ring-^{3}H]spiperone (19 Ci/mmol), 8-[dipropyl-2,3-^{3}H(*N*)]cyclopenthyl-1,3-dipropylxanthine (109 Ci/mmol), [ethylene-^{3}H]ketanserin hydrochloride (77 Ci/mmol), [methyl-^{3}H]tiotidine (84 Ci/mmol), and [7-methoxy-^{3}H]prazosin (72 Ci/mmol) were purchased from Du-Pont de Nemours (Brussels, Belgium). α-Modified Eagle's minimal essential medium (α-MEM), Dulbecco's phosphate-buffered saline, penicillin, gentamicin, streptomycin, fetal calf serum, and L-glutamine were bought from BioWhittaker (Verviers, Belgium). All other reagents were of analytical grade and obtained from conventional commercial sources.

Cloning and Site-Directed Mutagenesis

Cloning and stable expression of human histamine H_1 receptors in Chinese hamster ovary (CHO) cells were done in collaboration with Dr. A. Bollen (Department of Applied Genetics, Free University of Brussels, Brussels, Belgium) (Moguilevsky et al., 1994). To perform the mutagenesis, we used the human H_1 receptor cDNA cloned into the plasmid pRc/RSV (pNIV 3604) as template for the synthesis of DNA with site-specific mutations using a polymerase chain reaction strategy. For the introduction of the mutations Thr^{194} to Ala and Lys^{191} to Ala in the TM5, a 1286-bp *Dra*III-*Xba*I fragment from pNIV 3604 was isolated before amplification of a 147-bp internal region flanked by the sites *Xmn*I and *Bgl*II. After sequencing to ensure that there were no *Taq* polymerase-induced mutations, the *Xmn*I-*Bgl*II fragments carrying the mutations Thr^{194}→Ala and Lys^{191}→Ala were ligated with a 831-bp *Bgl*II-*Xba*I fragment, a 507-bp *Hin*dIII-*Xmn*I and the *Hin*dIII-*Xba*I fragment of the eukaryotic vectors pRc/RSV and pRc/CMV, leading to the recombinant plasmids pNIV 3608 and pNIV 3626, respectively. CHO cells (American Type Culture Collection, Manassas, VA), grown in 5% CO_2 at 37°C in α-MEM medium supplemented with 2 mM L-glutamine and 5% fetal calf serum, were transfected by electroporation using plasmids pNIV 3608 and pNIV 3626 (10 μg of DNA per 10^7 cells). Stably-transfected CHO cells were selected in medium containing Geneticin at 400 μg/ml. Resistant clones were isolated, subcloned, and expanded for subsequent $[^3H]$mepyramine binding assays.

Cell Culture and Membrane Preparation

CHO cells were subcultured in α-MEM medium containing 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 400 μg/ml Geneticin, and supplemented with 5% fetal calf serum. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air. Confluent cells were detached by a 10-min incubation in phosphate-buffered saline containing 1 mM EDTA. All subsequent operations were performed at 4°C. The cell suspension was centrifuged for 10 min at 500 *g*. The pellet was homogenized in 20 mM Tris-HCl, pH 7.4, 250 mM sucrose buffer, and frozen in liquid nitrogen. After thawing the homogenate was centrifuged at 30,000*g* for 15 min. The crude membrane pellet obtained was resuspended in the same buffer at a protein concentration of 6 to 8 mg/ml and stored in liquid nitrogen.

Equilibrium Binding Experiments

Equilibrium H_1 binding assays were performed as described by Moguilevsky et al. (1994) and in Table 1. For saturation binding isotherms, membranes (15 to 50 μg of proteins) from CHO cells expressing wild-type or mutant H_1 receptors were incubated with increasing concentrations of $[^3H]$mepyramine (from 0.2 to 20 nM). Binding experiments, at one drug concentration, were also carried out on a variety of other receptors or channels. Experimental conditions are listed in Table 1. Typically, after the incubation period, receptor-bound radioligand was separated from the free ligand by rapid vacuum filtration of the samples over GF/C glass fiber filters (Whatman, VEL, Belgium). Filters were presoaked in 0.1 to 0.3% polyethylenimine to reduce the nonspecific binding of the radioligand. Adsorbed samples were washed four times with 2 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4. The entire filtration procedure did not exceed 10 s/sample. Radioactivity trapped onto the filter was determined by liquid scintillation counting at 50 to 60% efficiency. For H_1 SPA binding assay, 500 μg of wheat germ agglutinin-coated SPA beads were incubated with 50 μg of membranes in 200 μl of 50 mM Tris HCl buffer, pH 7.4, containing 2 mM $MgCl_2$, 7.5 nM $[^3H]$mepyramine and increasing concentration of drugs. The 96-well microplates were centrifuged (5 min at 1000*g*), sealed, and counted at various intervals of time in a scintillation counter.

To determine whether the interactions between cetirizine or its enantiomers and histamine were competitive or allosteric, we used an experimental design based on the model described by Lazareno and Birdsall (1995). Briefly, competition curves between histamine and $[^3H]$mepyramine were carried out in the presence or absence of several concentrations of cetirizine or its enantiomers: each individual histamine binding curve was obtained in the presence of a single concentration of cetirizine or its enantiomers.

Kinetic Binding Experiments

Association. Binding was initiated by the addition of membranes to the incubation buffer containing 3.5 nM $[^3H]$mepyramine in the presence or absence of 10 μM cetirizine to define nonspecific binding. At increasing intervals of time thereafter, samples were filtered as described above.

Dissociation. Membranes were added to the incubation buffer containing 3.5 nM $[^3H]$mepyramine and binding was allowed to proceed for 60 min. At that time, radioligand dissociation was induced by the addition of cetirizine 10 μM. Sample aliquots were taken at increasing time intervals and filtered as explained above.

To determine the binding kinetics of unlabeled drugs to H_1 receptors, we measured the association kinetics of $[^3H]$mepyramine in the presence of a concentration of drug inhibiting by ±70% the specific binding of the radioligand at equilibrium.

Data Analysis

Data analysis was performed by computerized nonlinear curve fitting methods according to equations describing several binding models.

Competitive Interactions. Analysis of equilibrium data according to competitive interactions between labeled and unlabeled ligands which obey to the law of mass action (Molinoff et al., 1981). IC_{50} values were corrected to K_i values by applying the Cheng and Prusoff (1973) equation: $IC_{50} = K_i \times [1 + ([L] / K_D)]$, where IC_{50} is the concentration of unlabeled drug inhibiting by 50% the radioligand specific binding, [L] is the free radioligand concentration, and K_D and K_i are the equilibrium dissociation constants of the radioligand and unlabeled drug, respectively.

In the presence of a second unlabeled drug, the equation is extended to: $IC_{50} = K_i \times [1 + ([L] / K_D) + ([X] / K_X)]$, where [X] and K_X represent the concentration and equilibrium dissociation constant of the second unlabeled drug, respectively.

TABLE 1

Binding selectivity profile of cetirizine and its enantiomers

Receptor	Tissue	Radioligand			Assay Conditions			Inhibition of Radioligand Binding[b]		
		Name	K_D	Conc	Vol	Incubation	Buffer[a]	Cetirizine	Levocetirizine	(*S*)-Cetirizine
			nM	*nM*	*ml*				%	
A_1	Human, cloned	$[^3H]$DPCPX	1.8	0.2	0.5	60 min/25°C	B	0 ± 6	11 ± 6	8 ± 4
α_1	Rat cerebral cortex	$[^3H]$Prazosin	0.1	0.1	0.5	60 min/25°C	A	44 ± 8	57 ± 5	9 ± 6
α_2C4	Human, cloned	$[^3H]$RX821002	1.6	0.8	0.5	60 min/25°C	A	22 ± 2	29 ± 2	8 ± 3
β_1	Rat cerebral cortex	$[^3H]$CGP-12177	0.2	0.2	0.5	60 min/25°C	A	1 ± 8	4 ± 2	3 ± 2
D_1	Rat striatum	$[^3H]$SCH-23390	0.2	0.03	2.0	60 min/25°C	C	6 ± 6	9 ± 3	1 ± 1
D_2	Rat striatum	$[^3H]$Spiperone	0.04	0.1	2.0	120 min/25°C	C	2 ± 2	5 ± 6	−2 ± 1
H_1	Human, cloned	$[^3H]$Mepyramine	3.7	3.0	0.5	180 min/37°C	A	100 ± 1	100 ± 1	98 ± 1
H_2	Guinea pig cerebral cortex	$[^3H]$Tiotidine	4.8	5.0	0.25	60 min/25°C	A	15 ± 11	22 ± 13	15 ± 14
H_3	Guinea pig cerebral cortex	$[^3H]N^{\alpha}$MH	0.5	0.2	0.5	90 min/25°C	A	−2 ± 3	3 ± 1	6 ± 4
Muscarinic	Rat cerebral cortex	$[^3H]$NMS	0.06	0.1	1.0	60 min/25°C	A	6 ± 6	2 ± 1	4 ± 2
5-HT_{1A}	Rat hippocampus	$[^3H]$8-OH-DPAT	0.4	0.2	0.5	60 min/25°C	D	0 ± 3	−2 ± 4	−2 ± 1
5-HT_2	Rat cerebral cortex	$[^3H]$Ketanserin	0.5	0.3	1.0	60 min/25°C	A	32 ± 10	33 ± 1	7 ± 1
L-type Ca^{2+}	Rat cerebral cortex	$[^3H]$D888	2.3	0.3	0.5	60 min/25°C	A	16 ± 2	17 ± 5	13 ± 3
Na^+ type 1[c]	Rat cerebral cortex	$[^3H]$Saxitoxin		2.0		30 min/22°C		−4 ± 2	−1 ± 2	−4 ± 4
Na^+ type 2[c]	Rat cerebral cortex	$[^3H]$Batrachotoxinin		10.0		60 min/22°C		−3 ± 0	1 ± 2	−7 ± 16

[a] Buffer composition: A, 50 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$; B, A + 0.2 IU of adenosine deaminase; C, A + 0.01 μM ketanserin; D, A + 10 μM pargyline.

[b] Cetirizine and its enantiomers were tested at 10 μM and the results are the mean ± S.D. of three experiments.

[c] Results from studies subcontracted to CEREP (France).

Allosteric Interactions. Analysis of equilibrium data according to allosteric interactions of a molecule with labeled and unlabeled ligands (Lazareno and Birdsall, 1995),

$$\boxed{\begin{array}{ccccccc} & \mathrm{X} & & \mathrm{X} & & \mathrm{X} & \\ & + & \mathrm{K_L} & + & \mathrm{K_A} & + & \\ & \mathrm{LR} & \rightleftharpoons & \mathrm{L+R+A} & \rightleftharpoons & \mathrm{AR} & \\ \alpha\mathrm{K_x} & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright\ \mathrm{K_X} & & \upharpoonleft\downharpoonright & \beta\mathrm{K_x} \\ & \mathrm{LRX} & \rightleftharpoons & \mathrm{L+RX+A} & \rightleftharpoons & \mathrm{ARX} & \\ & & \alpha\mathrm{K_L} & & \beta\mathrm{K_A} & & \end{array}}$$

$$B = \mathrm{R_T}\frac{[\mathrm{L}]K_\mathrm{L}(1+\alpha[\mathrm{X}]K_\mathrm{X})}{1+[\mathrm{X}]K_\mathrm{X}+([\mathrm{A}]K_\mathrm{A})^{\mathrm{n}}(1+\beta[\mathrm{X}]K_\mathrm{X})+[\mathrm{L}]K_\mathrm{L}(1+\alpha[\mathrm{X}]K_\mathrm{X})}$$

where B = radioligand specific binding; $\mathrm{R_T}$ = total number of receptors; [L] = free radioligand concentration; [A] = free unlabeled agonist concentration; [X] = free unlabeled antagonist concentration; $\mathrm{K_L}$, $\mathrm{K_A}$, and $\mathrm{K_X}$ = the radioligand, agonist, and antagonist affinity constants; n = the Hill coefficient; α and β are the allosteric constants.

Kinetic Constants. Determination of the kinetic constants of unlabeled drugs according to a model described by Motulsky and Mahan (1984),

$$\boxed{\mathrm{R}+\mathrm{L}\underset{k_2}{\overset{k_1}{\rightleftharpoons}}\mathrm{RL} \quad \mathrm{R}+\mathrm{I}\underset{k_4}{\overset{k_3}{\rightleftharpoons}}\mathrm{RI}}$$

$$[RL]=\frac{\mathrm{N}k_1[\mathrm{L}]}{K_F-K_S}$$

$$\left[\frac{k_4(K_F-K_S)}{K_FK_S}+\frac{(k_4-K_F)}{K_F}\exp(-K_Ft)-\frac{(k_4-K_S)}{K_S}\exp(-K_St)\right]$$

$$K_F = 0.5[(K_A+K_B+\sqrt{(K_A-K_B)^2+4k_1k_3[\mathrm{L}][\mathrm{I}]})]$$

$$K_S = 0.5[(K_A+K_B-\sqrt{(K_A-K_B)^2+4k_1k_3[\mathrm{L}][\mathrm{I}]})]$$

$$K_A = k_1[\mathrm{L}]+k_2$$

$$K_B = k_3[\mathrm{I}]+k_4$$

where [L] = free radioligand concentration; [I] = free competitor concentration; r = free receptors; [RL] = radioligand specific binding; RI = competitor-receptor complex; N = R + RL + RI = total number of receptors; k_1, k_2 and k_3, k_4 are the radioligand and competitor association and dissociation rate constants, respectively.

Statistics. Partial F-tests were performed to compare two models (De Lean et al., 1982) and unpaired, two-tailed Student t tests were used to compare pKi or kinetic constants.

Results

Equilibrium Binding Experiments. Preliminary experiments indicated that competition curves with cetirizine and levocetirizine shifted to the left (decreasing the $\mathrm{IC_{50}}$ value) with time. This is exemplified in Fig. 2, which depicts an SPA binding assay in which the $\mathrm{IC_{50}}$ values of levocetirizine are clearly decreasing with time whereas those of (S)-cetirizine are time-independent, suggesting different binding kinetics for the two enantiomers. Therefore, although the [^{3}H]mepyramine equilibrates extremely rapidly, the incubation time was increased to 3 h to allow drugs with slow binding kinetics to reach steady state binding. As shown in Fig. 3 and Table 2, levocetirizine, the eutomer, has a 2-fold higher affinity than the racemic compound cetirizine ($p < 0.01$). The distomer, (S)-cetirizine, is about 30-fold less potent. The selectivity of cetirizine, levocetirizine, and (S)-cetirizine for $\mathrm{H_1}$ receptors, compared with a variety of GPCRs or channels that are known to bind first generation antihistamines, was evaluated (Table 1). No significant interactions were observed for any of the three compounds (tested at 10 μM), except for levocetirizine with the human α_{2C4} adrenergic receptor. The affinity of levocetirizine for these receptors (pK_i = 5.8 ± 0.1; n = 2) was still 600 times less than its affinity for $\mathrm{H_1}$ receptors.

We further characterized the binding of cetirizine and its enantiomers to $\mathrm{H_1}$ receptors by verifying the competitive nature of their interactions, not only with respect to [^{3}H]mepyramine but also to histamine. The $\mathrm{IC_{50}}$ of levocetirizine increased linearly with increasing concentrations of radioligand (Fig. 4). The K_i values obtained by applying the Cheng and Prusoff equation were independent of the radioligand concentration and were identical to the value obtained by linear regression of the data (intercept of the ordinate)

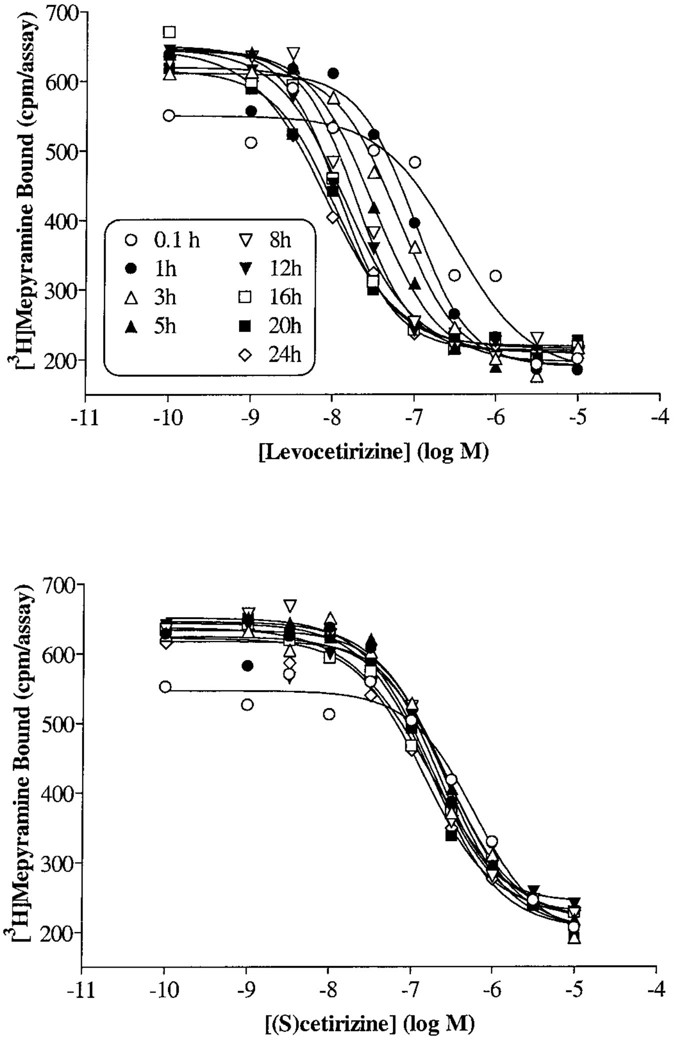

Fig. 2. Effect of incubation time on the $\mathrm{IC_{50}}$ of slowly and rapidly equilibrating drugs. Competition binding curves for levocetirizine and (S)-cetirizine were obtained after varying incubation times using an SPA binding assay. Incubations were started by adding 500 μg of SPA beads precoated with $\mathrm{H_1}$ receptors to samples in 96-well plates containing 7.5 nM [^{3}H]mepyramine and increasing concentrations of unlabeled drugs. Results are representative of two experiments.

suggesting a competitive behavior of the compound. The results for cetirizine and (*S*)-cetirizine were similar (data not shown). Competition curves between [^{3}H]mepyramine and histamine were performed in the presence or absence of given concentrations of cetirizine or its enantiomers. A representative set of curves is given for levocetirizine in Fig. 5, top. Histamine IC_{50} values increased linearly with increasing concentrations of levocetirizine, as would be expected for two compounds that interact competitively at a single binding site (Fig. 5, bottom). By analyzing the data with the allosteric/competitive ternary complex model (Lazareno and Birdsall, 1995), we found values for α (representing the interaction between cetirizine or its enantiomers with [^{3}H]mepyramine) equal to 0 for all three compounds, whereas values for β (representing the interaction between cetirizine or its enantiomers with histamine) were equal to 0.07 ($n = 1$), 0.12 ± 0.06 ($n = 3$), and 0.07 ($n = 1$) for cetirizine, levocetirizine, and (*S*)-cetirizine, respectively, suggesting strong negative allosteric interactions, very close to a competitive behavior. Partial F-tests performed to compare the competitive model (with the allosteric constants set to 0) and the allosteric model indicated that the data were not better fitted with the allosteric model ($p > 0.15$ except for one of three experiments performed with levocetirizine where $p < 0.05$).

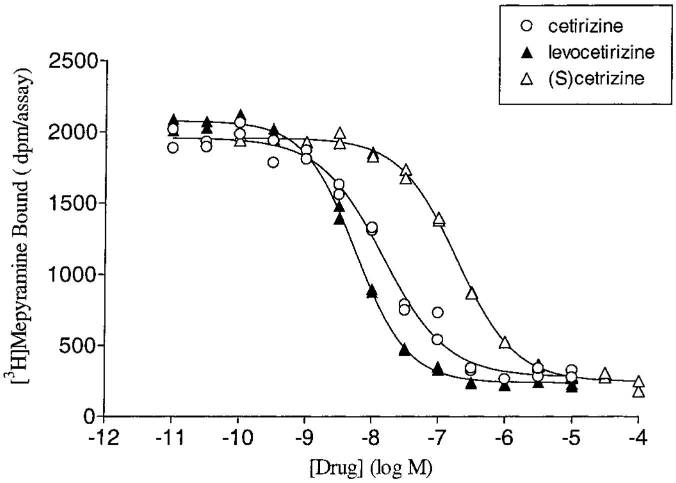

Fig. 3. Affinity of cetirizine and its enantiomers for cloned human H_1 histamine receptors. Membranes from CHO cells expressing cloned human H_1 histamine receptors were incubated for 3 h at 37°C with increasing concentrations of compounds as described under *Materials and Methods*. The binding curves presented are representative of at least three experiments. Duplicates obtained for each concentration are visible on the graph.

Kinetic Binding Experiments. Association kinetics of [^{3}H]mepyramine were performed in the absence and presence of a single concentration of unlabeled drug producing ± 70% inhibition of the radioligand specific binding at equilibrium. Data were analyzed according to the model of Motulsky and Mahan (1984) describing the kinetics of competitive radioligand binding as predicted by the law of mass action. The kinetic constants of [^{3}H]mepyramine and the amount of receptors in the membrane preparations were determined independently and were kept constant to analyze the data in the presence of the unlabeled drug. It is clear from Fig. 6 that (*S*)-cetirizine reaches equilibrium faster than levocetirizine at equiactive concentrations. Indeed, the analysis shows that although levocetirizine and (*S*)-cetirizine have quite similar association constants, they differ strikingly when considering the dissociation constants; levocetirizine has a half-time of dissociation longer than 2 h compared with only 6 min for (*S*)-cetirizine (Table 3). It is noteworthy to point out that the pK_i values calculated from the ratio of the two kinetic constants k_{-1}/k_{+1} (8.7 ± 0.1 and 7.1 ± 0.1, respectively) agree perfectly with those observed experimentally at equilibrium (8.5 ± 0.1 and 7.1 ± 0.1, respectively). We also looked at the binding kinetics of close analogs of cetirizine, whose only structural differences reside in the carboxyl group being replaced either by an hydroxyl group or by a methyl ester (Fig.

TABLE 2

Affinity of compounds for wild-type and mutant H_1 receptors

Results are expressed as $-\log K_i$ and are the mean ± S.D. of three experiments. Experiments were carried out for 3 h at 37°C and K_i values were calculated from IC_{50} as explained under *Materials and Methods. P* values were obtained from two-tailed, unpaired Student *t* tests comparing results from mutant receptors with those of wild-type receptors.

Compounds	Wild-Type		$Lys^{191} \rightarrow Ala^{191}$		$Thr^{194} \rightarrow Ala^{194}$	
	pK_i	n_H	pK_i	n_H	pK_i	n_H
Histamine	5.9 ± 0.0	0.74 ± 0.07	4.7 ± 0.1***	0.74 ± 0.19	5.2 ± 0.1***	0.75 ± 0.03
Cetirizine	8.2 ± 0.1	1.06 ± 0.12	N.D.		N.D.	
Levocetirizine	8.5 ± 0.1†	1.09 ± 0.07	7.9 ± 0.1***	1.05 ± 0.24	8.7 ± 0.1*	1.29 ± 0.09
(*S*)-Cetirizine	7.1 ± 0.1	0.96 ± 0.04	6.3 ± 0.0***	0.97 ± 0.08	8.2 ± 0.2***	0.97 ± 0.02
Hydroxyzine	8.7 ± 0.1	1.07 ± 0.10	N.D.		N.D.	
(*S*)-Hydroxyzine	7.5 ± 0.1	1.07 ± 0.02	N.D.		8.4 ± 0.0***	1.17 ± 0.10
(*R*)-Hydroxyzine	9.0 ± 0.1	1.08 ± 0.15	9.1 ± 0.0	1.07 ± 0.02	9.3 ± 0.0**	1.05 ± 0.12
(*R*)-ucb 29992	8.3 ± 0.2	1.19 ± 0.13	8.3 ± 0.2	1.02 ± 0.26	8.9 ± 0.1**	1.06 ± 0.18
(*S*)-ucb 29993	7.2 ± 0.1	1.14 ± 0.33	N.D.		8.1 ± 0.0***	0.94 ± 0.08
(+)-Chlorpheniramine	8.6 ± 0.1	1.01 ± 0.06	N.D.		N.D.	
Terfenadine	8.7 ± 0.0	1.09 ± 0.07	8.5 ± 0.1*	1.52 ± 0.19	N.D.	
Fexofenadine	8.0 ± 0.3	0.99 ± 0.27	7.4 ± 0.2*	0.90 ± 0.14	N.D.	
Loratadine	7.8 ± 0.0	0.97 ± 0.02	7.8 ± 0.3	1.03 ± 0.17	7.3 ± 0.0***	1.43 ± 0.08
[^{3}H]Mepyramine						
K_D (nM)	4.2 ± 0.6		1.3 ± 0.1		0.9 ± 0.2	
B_{max} (fmol/mg of protein)	1367 ± 120		754 ± 234		334 ± 137	

N.D., not determined; n_H, Hill coefficient.
*, $p < 0.05$.
**, $p < 0.01$.
***, $p < 0.001$.
† $p < 0.05$ compared with cetirizine.

1). Although all these pairs of enantiomers have quite similar affinities for the H_1 receptor (Table 2), they differ strikingly from a kinetic point of view (Table 3). Indeed, replacement of the carboxyl moiety leads to a sharp increase in the association rate (from 10- to 30-fold) but also, concomitantly, to an increase in the dissociation rate (from 4- to 20-fold).

Mutagenesis Experiments. Two mutants were of particular interest: the Thr^{194} to Ala mutation, which we showed was important for the stereoselectivity of cetirizine enantiomers (Moguilevsky et al., 1995). Here we extend our previous observations on Thr^{194} by including other pairs of enantiomers and by studying the effects of this mutation on the kinetic constants of the molecules. The second mutant is Lys^{191} to Ala, which might theoretically be important for the recognition of the carboxyl group of cetirizine and its enantiomers. The pK_i and kinetic constants are reported in Table 2 and 3, respectively.

Mutation Thr^{194}→Ala. The results are compiled in Table 2 and 3. [^{3}H]Mepyramine bound with higher affinity to this mutant than to the wild-type receptor. The mutation provoked an 8- to 13-fold increase in the affinity of the distomers [i.e., (*S*)-cetirizine, (*S*)-hydroxyzine, and (*S*)-ucb 29993]. The increase in affinity of the corresponding eutomers was limited to 1.5- to 4-fold. As a consequence, the binding stereoselectivity of the enantiomers was reduced with eudismic indexes decreasing from 25 to 3 for levocetirizine and (*S*)-cetirizine, from 32 to 8 for (*R*)-hydroxyzine and (*S*)-hydroxyzine and from 13 to 6 for (*R*)-ucb 29992 and (*S*)-ucb 29993. Histamine and loratadine, on the contrary, had 3- to 5-fold lower affinity for the mutant receptor. On a kinetic level, levocetirizine and (*S*)-cetirizine experienced both an increase in their k_{+1} but only (*S*)-cetirizine had a concomitant decrease in its k_{-1}. The same observation applies for the couple (*R*)- and (*S*)-hydroxyzine. As for (*R*)-ucb 29992

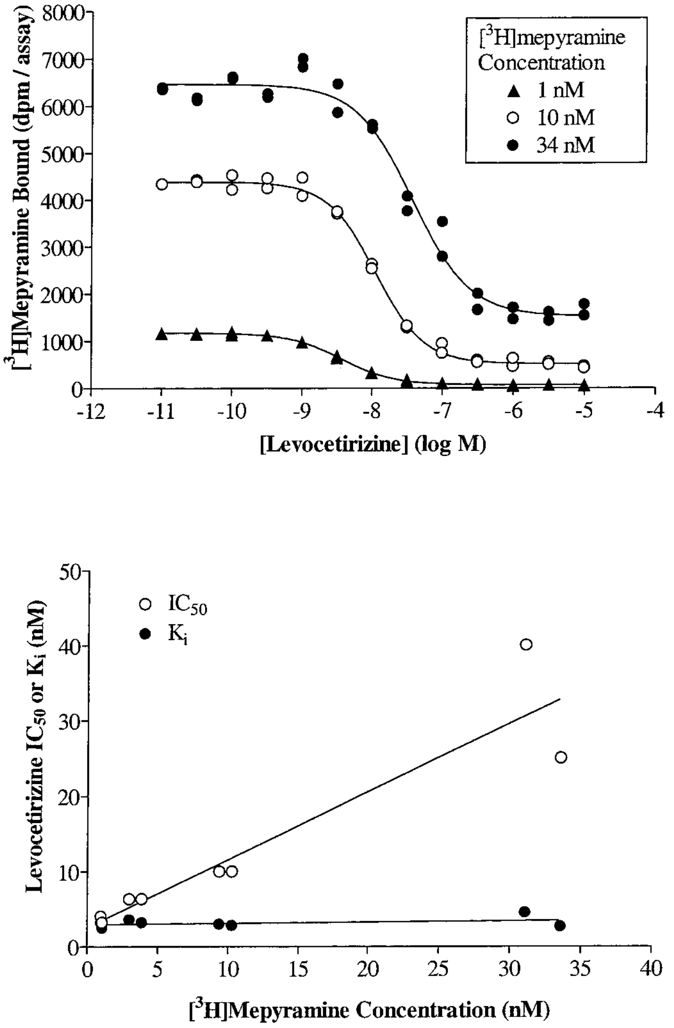

Fig. 4. Cetirizine and its enantiomers interact competitively with [^{3}H]mepyramine. The concept is exemplified in this figure for levocetirizine. Similar results were obtained for cetirizine and (*S*)-cetirizine. Top, competition binding curves between levocetirizine and [^{3}H]mepyramine were obtained at increasing concentrations of radioligand. The curves presented are representative of two experiments performed in duplicate. Bottom, the IC_{50} values calculated by nonlinear regression analysis of all binding curves were plotted against the concentrations of radioligand. K_i values were calculated by transforming the IC_{50} values according to the Cheng and Prusoff equation as described under *Materials and Methods*.

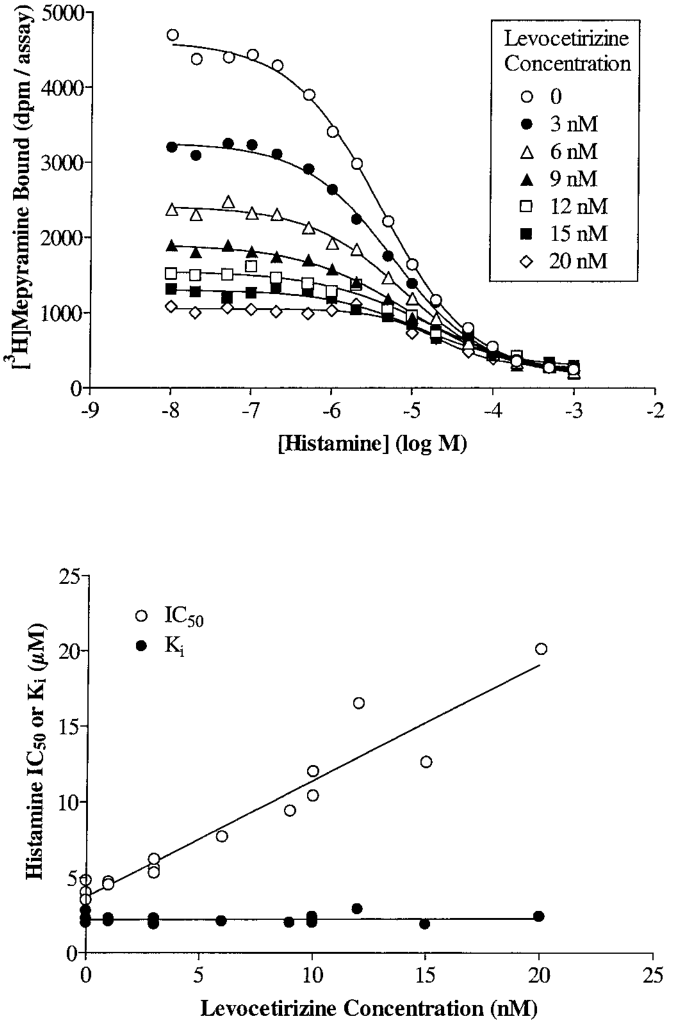

Fig. 5. Cetirizine and its enantiomers interact competitively with histamine. The concept is exemplified in this figure for levocetirizine. Similar results were obtained for cetirizine and (*S*)-cetirizine. Top, competition binding curves between histamine and [^{3}H]mepyramine in the presence of increasing concentrations of levocetirizine. The curves presented are representative of two experiments. Bottom, the IC_{50} values calculated for histamine by nonlinear regression analysis of the binding curves were plotted against the concentrations of levocetirizine. K_i for histamine were calculated by transforming the IC_{50} values according to the modified Cheng and Prusoff equation as described under *Materials and Methods*.

and (*S*)-ucb 29993, there is a similar trend, although it is not statistically significant because of the rather large variations in the kinetic constants calculated for compounds having very fast kinetics.

Mutation Lys^{191}→Ala. The results are compiled in Table 2 and 3. The binding of [^{3}H]mepyramine was not significantly affected by this mutation whereas the affinity of histamine was decreased by 20-fold. At equilibrium, the affinity of levocetirizine and (*S*)-cetirizine for the mutant receptor was decreased by a factor of 4 to 6, whereas the affinity of the hydroxyl or methyl ester analogs was hardly changed. Terfenadine and fexofenadine (the carboxyl derivative of terfenadine) also experienced a slight decrease in affinity (about 2 to 4 fold), whereas that of loratadine remained unchanged. On a kinetic level, the association rates of all compounds increased by 2- to 5-fold, except for (*R*)-ucb 29992, for which no significant changes were observed. By contrast, the dissociation rates for levocetirizine and (*S*)-cetirizine were increased by 10- and 6-fold, respectively compared with only 2-fold for the hydroxyl analog (*R*)-hydroxyzine and with no change for the ester analog (*R*)-ucb 29992. Both terfenadine and fexofenadine showed a similar 3-fold increase in their dissociation rates whereas a 3-fold decrease was observed for loratadine.

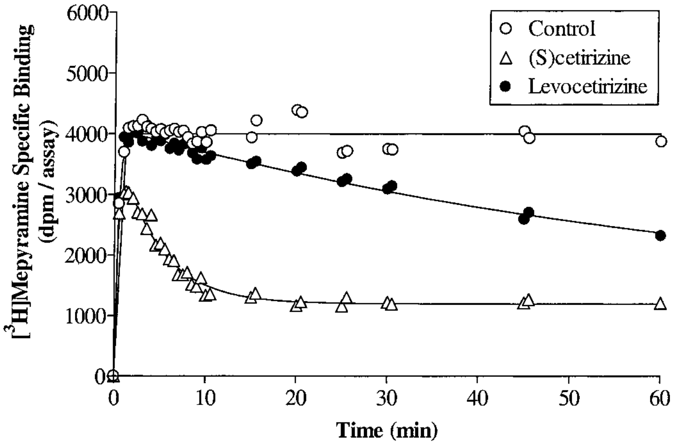

Fig. 6. Determination of the binding kinetic constants of levocetirizine and (*S*)-cetirizine. [^{3}H]Mepyramine association kinetics were performed in the presence or absence of a single concentration of levocetirizine or (*S*)-cetirizine (chosen to inhibit approximately 75% of [^{3}H]mepyramine specific binding at equilibrium). Data were analyzed according to the model proposed by Motulsky and Mahan (1984) as described under *Materials and Methods*. The curves are the actual fitting and are representative of three experiments.

Discussion

Cetirizine is a second generation antihistamine drug, displaying high affinity and selectivity for cloned human H_1 histamine receptors. As we showed previously, cetirizine and its enantiomers levocetirizine and (*S*)-cetirizine bind stereoselectively to this receptor with a eudismic ratio of 30 in favor of levocetirizine (Moguilevsky et al., 1994, 1995). Here, we characterize in more detail the molecular interactions of these three compounds with the human H_1 receptor. First, it seems clear from the results that the two enantiomers bind to the receptors with quite different kinetics; although they have quite similar association constants, their dissociation rates are different, with levocetirizine dissociating from the receptors with a half-time of 142 min compared with only 6 min for (*S*)-cetirizine. The difference in dissociation rates between these compounds accounts for most of the difference in their affinities. The dissociation half-time found for levocetirizine agrees well with the 130 min measured for cetirizine on guinea pig H_1 receptors using another method (Leysen et al., 1991). One practical consideration about long dissociation kinetics is the time needed to reach equilibrium in binding or other in vitro experiments. Short incubation times will lead to underestimation of the affinity of slowly equilibrating drugs, as exemplified in the SPA binding assay. With time, levocetirizine competition curves shifted to the left along the concentration axis giving decreasing IC_{50} values from 300 nM at 10 min to 10 nM after 8 h incubation, whereas IC_{50} values for (*S*)-cetirizine decreased only from 500 nM to 250 nM in the same interval of time, as expected for a compound that dissociates much faster and thus reaches equilibrium more quickly. As a consequence, the stereoselectivity ratio for such compounds will depend on the incubation time, going from approximately 1.5 after 6 min to 25 after 8 h.

Cetirizine has been reported as acting as a noncompetitive

TABLE 3

Binding kinetic constants of antagonists to wild-type and mutant H_1 receptors

Results are the mean ± S.D. of three experiments. Data were analyzed according to the model proposed by Motulsky and Mahan (1984) as described under *Materials and Methods*. k_{-1} for [^{3}H]mepyramine were measured directly in separate experiments and kept constant in the model. *P* values were obtained from two-tailed, unpaired Student *t* tests comparing results from mutant receptors with those of wild-type receptors.

	Wild-Type			$Lys^{191} \rightarrow Ala^{191}$			$Thr^{194} \rightarrow Ala^{194}$		
	$k_{+1}{}^a$	$k_{-1}{}^b$	$t_{1/2}{}^c$	$k_{+1}{}^a$	$k_{-1}{}^b$	$t_{1/2}{}^c$	$k_{+1}{}^a$	$k_{-1}{}^b$	$t_{1/2}{}^c$
[^{3}H]Mepyramine	513 ± 186	0.86 ± 0.08	0.8	923 ± 204	1.10 ± 0.04**	0.6	443 ± 40	0.21 ± 0.02***	4
Levocetirizine	2.3 ± 0.4	0.005 ± 0.002	142	5.9 ± 1.2**	0.052 ± 0.026*	13	7.1 ± 1.8**	0.007 ± 0.002	107
(*S*)-Cetirizine	1.6 ± 0.7	0.12 ± 0.05	6	4.6 ± 1.4*	0.69 ± 0.29*	1.0	5.0 ± 1.3**	0.030 ± 0.013*	24
(*S*)-Hydroxyzine	25 ± 2	0.47 ± 0.15	1.5	N.D.	N.D.	N.D.	73 ± 52	0.15 ± 0.10*	5
(*R*)-Hydroxyzine	13 ± 2	0.022 ± 0.006	31	66 ± 21*	0.042 ± 0.008*	17	66 ± 15**	0.015 ± 0.004	47
(*R*)-ucb 29992	70 ± 12	0.097 ± 0.04	7	81 ± 9	0.070 ± 0.013	10	131 ± 41	0.088 ± 0.044	8
(*S*)-ucb 29993	83 ± 32	3.313 ± 2.01	0.2	N.D.	N.D.	N.D.	180 ± 166	0.87 ± 0.52	0.8
Terfenadine	10 ± 5	0.019 ± 0.009	37	30 ± 11*	0.054 ± 0.024*	13	N.D.	N.D.	N.D.
Fexofenadine	1.2 ± 0.3	0.011 ± 0.004	62	2.2 ± 0.6*	0.031 ± 0.006**	22	N.D.	N.D.	N.D.
Loratadine	3.6 ± 0.6	0.13 ± 0.02	5	5.8 ± 0.2*	0.045 ± 0.013*	15	N.D.	N.D.	N.D.

[a], Association kinetic constants are expressed in $min^{-1} \times 1 \times \mu mol^{-1}$.
[b], Dissociation kinetic constants are expressed in min^{-1}.
[c], Dissociation half-time is expressed in min and was calculated as ln $2/k_{-1}$.
*, $p < 0.05$.
**, $p < 0.01$.
***, $p < 0.001$.

antagonist when inhibiting in vitro histamine-induced contractions of human bronchus (Advenier et al., 1991) and guinea pig trachea (Kahler and Du Plooy, 1994). These observations can now be easily explained by the slow dissociation kinetic of levocetirizine (which, as the eutomer, is the active component of cetirizine). Indeed, slowly dissociating drugs may virtually act as irreversible antagonists and produce what is now called insurmountable antagonism in functional studies; i.e., the maximal tissue response produced by the agonist will be depressed at high antagonist concentration (Kenakin, 1993; see Jenkinson et al., 1995, for nomenclature). Insurmountable antagonism related to slow dissociation kinetics has also been reported for AT_2 antagonists (Olins et al., 1995). The extent of insurmountable antagonism caused by slowly dissociating drugs will also depend on the receptor reserve present in the tissue under study as illustrated with cetirizine and levocetirizine in guinea pig trachea and ileum (Christophe et al., 2000). Although interactions with L-type calcium channels can also cause insurmountable antagonism in the same experimental settings, we have shown that cetirizine and levocetirizine, up to 10 μM, did not interact with these channels. We also have shown in this study that cetirizine and both enantiomers interact competitively with histamine at the receptor level. Indeed, the three compounds increased, in a dose-dependent manner, the IC_{50} of histamine in competition binding assays as expected for competitive antagonists (Fig. 5). When we analyzed the data according to an allosteric model (Lazareno and Birdsall, 1995), best fits were obtained with allosteric constants close to or equal to 0, indicative of strong negative allosterism or competitive antagonism. Negative allosterism implies that in the presence of the antagonist, the agonist will still be able to bind to the receptor, albeit with a lower affinity. In this regard, competitive antagonism can be viewed as an extreme case of allosteric antagonism in which the agonist affinity is reduced to 0, making competitive and strongly negative allosteric antagonists quite difficult to distinguish (Ehlert, 1988).

The carboxyl group of cetirizine or levocetirizine, which is ionized at physiological pH (Pagliara et al., 1998), although not important for the affinity of the compounds, is responsible for the long dissociation time. Its replacement by a hydroxyl group or a methyl ester group hardly modifies the affinity but increases both the dissociation and association kinetic constants at the H_1 receptor. The dissociation half-time decreases from 142 min for levocetirizine to 31 min for (*R*)-hydroxyzine (the hydroxyl analog) and 7 min for (*R*)-ucb 29992 (the methyl ester analog). A comparable effect was observed with the corresponding distomers.

The mutation of Lys^{200} into Ala in the guinea pig H_1 receptor was first reported to lead to a decrease in histamine affinity without much change in antagonist affinity (Leurs et al., 1995). However, a second study by the same group showed that with antagonists bearing a carboxyl group, their affinity falls by 8- to 50-fold (Wieland et al., 1999). The human counterpart of the guinea pig Lys^{200} is located in position 191 of the fifth transmembrane domain. We mutated Lys^{191} into Ala and observed, as previously published for the guinea pig, a 20-fold lower affinity of histamine for this receptor compared with the wild-type receptor. More interesting, however, was the observation that cetirizine and its enantiomers also had a reduction in affinity between 3- and 5-fold, whereas the affinity of their structural analogs lacking the carboxyl group was unchanged. When looking at the kinetic constants, the picture is even clearer; if the mutation of Lys^{191} to Ala slightly (by 2-fold) increases the association constants, it had a much more pronounced effect on the dissociation rate, which was increased by a factor of 10, decreasing the dissociation half-life from 142 min to 13 min for levocetirizine. The hydroxyl analog [(*R*)-hydroxyzine] is much less sensitive to this mutation and its dissociation half-life was shortened by only 50%, whereas the mutation has no effect at all on the binding kinetics of the methyl ester analog [(*R*)-ucb 29992]. These results advocate for a strong interaction between the carboxyl moiety of cetirizine or its enantiomers and Lys^{191} of the human H_1 receptors and indicate that this interaction is the key to the slow dissociation rates of these compounds. The lesser effect of the mutation on the hydroxyl analog is in line with the weaker energy of the hydrogen bond that can still occur between the primary amine of the lysine and the hydroxyl group of the compound compared with the ionic bond expected with the carboxyl group. However, these results obtained on Lys^{191} with levocetirizine and by Wieland et al. (1999) with cetirizine and acrivastine cannot be extended to all second-generation antihistamines bearing a carboxyl group. Indeed, fexofenadine, the carboxyl analog of terfenadine, and terfenadine are equally sensitive to the mutation of Lys^{191}. A possible explanation might be the distance between the protonated nitrogen, believed to interact strongly with Asp^{107} (or Asp^{116} in the guinea pig) in the third transmembrane region and the carboxyl function. This distance is far greater in fexofenadine compared with cetirizine and places the carboxyl function of the former out of reach of any interaction with Lys^{191}. The hydroxyl group present in both terfenadine and fexofenadine, however, could be at the right distance to make an hydrogen bond with Lys^{191}. Alternatively, a rather hydrophobic environment (Moguilevsky et al., 1994) could stabilize a π cationic bond between the benzyl ring of terfenadine and fexofenadine and the nitrogen of Lys^{191}.

While we were studying the influence of Thr^{194} on the binding of histamine, for which we observed a 5-fold decrease in affinity for the mutant as reported by others (Leurs et al., 1994; Ohta et al., 1994), we also found, surprisingly, that the mutation of Thr^{194} into Ala decreased the stereoselectivity of cetirizine enantiomers (Moguilevsky et al., 1995). Although the mutation increases the affinity of both enantiomers, it is more pronounced for (*S*)-cetirizine, leading to an 8-fold decrease in stereoselectivity. The other enantiomeric pairs were also sensitive to this mutation but the stereoselectivity decrease was limited to 4-fold for (*R*)- and (*S*)-hydroxyzine and to 2-fold for (*R*)-ucb 29992 and (*S*)-ucb 29993. Because the chiral centers are identical in the three pairs of compounds, we could speculate that the interactions taking place with other amino acids, like Lys^{191}, are influencing the way the compounds are hindered by Thr^{194}.

In conclusion, we have shown in this study that cetirizine and levocetirizine are high-affinity, selective H_1 antagonists (more than 600-fold compared with a variety of other G-protein-coupled receptors and channels) interacting competitively with histamine. The eutomer levocetirizine has 2-fold higher affinity for H_1 receptors compared with cetirizine, the racemic compound. Its high affinity is related to slow dissociation kinetics, partly because of an interaction between the carboxylic moiety and Lys[191] of the human H_1 receptor. This slow dissociation rate also helps explain the insurmountable antagonism observed in certain functional assays. Finally, the 30-fold binding stereoselectivity of the enantiomers is, to some extent, the consequence of a hindrance caused by Thr[194].

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