

Pharmacological Differences between Human and Guinea Pig Histamine H₁ Receptors: Asn⁸⁴ (2.61) as Key Residue within an Additional Binding Pocket in the H₁ Receptor

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ABSTRACT

We tested several histamine H₁ receptor (H₁R) agonists and antagonists for their differences in binding affinities between human and guinea pig H₁Rs transiently expressed in African green monkey kidney (COS-7) cells. Especially, the bivalent agonist histaprodifen-histamine dimer (HP-HA) shows a higher affinity for guinea pig than for human H₁Rs. Based on the structure of HP-HA, we have further identified VUF 4669 [7-(3-(4-(hydroxydiphenylmethyl)piperidin-1-yl)propoxy)-4-oxochroman-2-carboxylic acid] as a guinea pig-preferring H₁R antagonist, demonstrating that the concept of species selectivity is not limited to agonists. To delineate the molecular mechanisms

behind the observed species selectivity, we have created mutant human H₁Rs in which amino acids were individually replaced by their guinea pig H₁R counterparts. Residue Asn⁸⁴ (2.61) in transmembrane domain (TM) 2 seemed to act as a selectivity switch in the H₁R. Molecular modeling and site-directed mutagenesis studies suggest that Asn⁸⁴ interacts with the conserved Tyr⁴⁵⁸ (7.43) in TM7. Our data provide the first evidence that for some H₁R ligands, the binding pocket is not only limited to TMs 3, 4, 5, and 6 but also comprises an additional pocket formed by TMs 2 and 7.

The biogenic amine histamine exerts its effects through binding and activation of four G protein-coupled receptors (GPCRs), the H₁, H₂, H₃, and H₄ receptors. The H₁ receptor (H₁R) regulates inflammatory and allergic responses and is successfully targeted by various drugs. H₁R antagonists have been on the market since 1942 for the treatment of allergies, and newer, non-sedating second generation H₁R antagonists are still the medication of choice to relieve certain allergic symptoms (Hill et al., 1997).

In contrast to the development of various potent H₁R antagonists, the synthesis of selective and potent H₁R agonists has not achieved the same success (Hill et al., 1997). Only in 1995, 2-(3-(trifluoromethylphenyl)histamine was discovered as the first selective H₁R agonist with a potency equal to histamine as determined by the H₁R-mediated guinea pig

ileum contractions (Leschke et al., 1995; Zingel et al., 1995). Recently, Elz et al. (2000) synthesized a series of compounds constituting a new class of highly active H₁R agonists, the histaprodifens. Histaprodifen combines a histamine moiety linked at the 2-position with an ω,ω -diphenylalkyl substituent, a characteristic of the H₁R antagonist pharmacophore (ter Laak et al., 1995; Zhang et al., 1997). Based on this new H₁R agonist, “dimeric” histaprodifens were subsequently developed, consisting of a histaprodifen moiety linked at the N α position to another histamine moiety, for example (histaprodifen-histamine dimer, HP-HA) (Menghin et al., 2003). Compared with histamine, the potency of HP-HA is reported to be 36- to 56-fold and 630-fold higher on guinea pig isolated ileum and trachea, respectively (Christophe et al., 2003; Seifert et al., 2003).

Contrary to the potencies at either the guinea pig ileum and trachea or rat aorta (Elz et al., 2000; Christophe et al., 2003; Seifert et al., 2003), the potencies of various histaprodifen analogs (histaprodifen, MeHP, HP-HA, and HP-HP) at

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ABBREVIATIONS: GPCR, G protein-coupled receptor; H₁R, histamine H₁ receptor; HP-HA, histaprodifen-histamine dimer; HP-HP, histaprodifen-histaprodifen dimer; MeHP, N α -methylhistaprodifen; TM, transmembrane domain; hH₁R, human histamine H₁ receptor; VUF 4669, 7-(3-(4-(hydroxydiphenylmethyl)piperidin-1-yl)propoxy)-4-oxochroman-2-carboxylic acid; VUF 8401, 3-(1*H*-imidazol-4-yl)propyl-3-(2-(benzhydrylthio)ethyl)guanidine dipicrate; 5HT, 5-hydroxytryptamine (serotonin); WT, wild type; gpH₁R, guinea pig histamine H₁ receptor.

human H₁Rs are at best similar to the potency of the endogenous ligand histamine (Seifert et al., 2003; Bruysters et al., 2004), indicating a potential species difference at the level of the H₁R recognition of these H₁R agonists.

In aminergic GPCRs, including the H₁R, the ligand-binding pocket is thought to reside in a hydrophilic cleft formed by the seven transmembrane domains (TMs). Within the third TM (TM3), an aspartate (Asp) residue is a conserved feature among these aminergic GPCRs and is likely to make a direct contact with the protonated amine of aminergic ligands (Shi and Javitch, 2002). Indeed, in the human H₁R Asp¹⁰⁷ in TM3 (residue 3.32 according to the Ballesteros-Weinstein numbering) is part of the binding pocket of both H₁R agonists and antagonists (Ohta et al., 1994; Moguilevsky et al., 1998; Nonaka et al., 1998; Bruysters et al., 2004). Several additional amino acids in TM5 and TM6 are part of the H₁R binding pocket of histamine: Lys¹⁹¹ (5.39) (Leurs et al., 1995; Moguilevsky et al., 1998; Wieland et al., 1999; Gillard et al., 2002; Bruysters et al., 2004), Asn¹⁹⁸ (5.46) (Leurs et al., 1994; Ohta et al., 1994; Moguilevsky et al., 1995; Bruysters et al., 2004), and Phe⁴³⁵ (6.55) (Bruysters et al., 2004) are considered to accommodate the imidazole ring of histamine. The H₁R antagonist binding pocket stretches deeper into the receptor protein and comprises the aromatic amino acids Trp¹⁵⁸ (4.56) (Wieland et al., 1999) and Phe⁴³² (6.52) (Wieland et al., 1999; Bruysters et al., 2004).

Recently, we studied the binding pocket of several histaprodifen analogs in the human H₁R (Bruysters et al., 2004). We demonstrated that histamine and the histamine moiety of histaprodifens bind to the human H₁R in a similar orientation. Although the diphenylalkyl system of histaprodifen interacts with the H₁R in an "antagonistic binding mode", i.e., interacting with Phe⁴³² (6.52) in TM6 (Bruysters et al., 2004), no interactions with Lys¹⁹¹ (5.39) and Phe⁴³⁵ (6.55) were found. Again, the interaction with both Asp¹⁰⁷ (3.32) proved crucial. Although Asn¹⁹⁸ (5.46) did not affect histaprodifen affinity, it seemed pivotal for agonist-induced activation of the hH₁R. An interaction between Asn¹⁹⁸ (5.46) and histaprodifen was therefore suggested (Bruysters et al., 2004).

We explored in this study the molecular basis of the observed species differences between human and guinea pig H₁Rs by a combined approach of molecular modeling and site-directed mutagenesis. We reevaluated several H₁R agonists and antagonists for their differences in affinity between human and guinea pig H₁Rs by [³H]mepyramine displacement studies. Based on our knowledge of the H₁R binding site of the histaprodifens and the high (93%) level of sequence homology within the TM domains of the human and guinea pig H₁Rs, we extended our approach to mutant human H₁Rs in which selected amino acids were individually replaced by their guinea pig H₁R counterparts. Using this strategy, we identified Asn⁸⁴ (2.61) in TM2 as the molecular basis for the observed species selectivity of certain H₁R ligands and discuss the implications of these findings for future drug design.

Materials and Methods

Chemicals. Chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, mepyramine (pyrilamine maleate), astemizole, ketotifen fumarate, 8*R*-lisuride, and terfenadine were purchased from Sigma-Aldrich (Bornem, Belgium). Oxatamide

was obtained from MP Biomedicals (Zoetermeer, The Netherlands). Fexofenadine was purchased from Ultrafine Chemicals (Manchester, UK). VUF 4669 and VUF 8401 were synthesized at the Vrije Universiteit Amsterdam (Amsterdam, The Netherlands). Cetirizine dihydrochloride (Zyrtec) and loratadine were synthesized at UCB S.A. (Braine l'Alleud, Belgium).

Gifts of 2-(3-trifluoromethylphenyl)histamine dihydrogen maleate, histaprodifen (2-[2-(3,3-diphenylpropyl)imidazol-4-yl]ethanamine dihydrogen maleate), methylhistaprodifen (*N*α-methyl-histaprodifen dihydrogen oxalate), histaprodifen-histaprodifen dimer trihydrogenoxalate and histaprodifen-histamine dimer (*N*α-[2-(1*H*-imidazol-4-yl)-ethyl]-histaprodifen) trihydrogenoxalate (Dr. W. Schunack, Free University, Berlin, Germany), the cDNA encoding the human H₁R (Dr. H. Fukui, University of Tokushima, Tokushima, Japan), and the expression vector pcDEF₃ (Goldman et al., 1996) (Dr. J. Langer, Robert Wood Johnson Medical School, Piscataway, NJ) are greatly appreciated.

Cell culture media, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium). Cell culture plastics were obtained from Greiner Bio-one (Wemmel, Belgium). [³H]Mepyramine (~20 Ci/mmol) was from Amersham Biosciences Inc. (Roosendaal, The Netherlands).

Numbering Scheme of GPCRs. The indexing method introduced by Ballesteros and Weinstein (1995) was used throughout to identify amino acids in the TM regions. Each residue is identified by two numbers: the first number corresponds to the helix (1–7) in which the residue is located, and the second number indicates its position relative to the most conserved amino acid in that helix, arbitrarily assigned to 50. Numbers depicted in superscript correspond to the number of the amino acid in the human H₁R.

Site-Directed Mutagenesis. The cDNA encoding the human H₁R (Fukui et al., 1994) was subcloned in the pAlter plasmid (Promega, Madison, WI), and point mutations were created according to manufacturer's protocol (Altered Sites II; Promega). cDNAs of mutant and wild-type receptors were subcloned into the expression plasmid pcDEF₃ (Goldman et al., 1996). Sequences were verified by DNA sequencing using the dideoxy chain termination method.

Cell Culture, Transfection, and Membrane Preparation. COS-7 African green monkey kidney cells were maintained at 37°C in a humidified 5% CO₂, 95% air atmosphere in Dulbecco's modified Eagle's medium containing 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal bovine serum. COS-7 cells were transiently transfected using the DEAE-dextran method as described previously (Bakker et al., 2001), by using 5 μg of plasmid DNA per million cells. Two days after transfection, cells were detached by scraping and were harvested by centrifugation. Cell pellets were resuspended in ice-cold water, lysed by repetitive freezing/thawing, and frozen in liquid nitrogen. The obtained crude cell homogenates were stored at –80°C until further use.

H₁R Binding Studies. The COS-7 cell homogenates (~5 μg) were incubated for 60 min at 30°C in 500 μl of binding buffer (50 mM Na₂/K-phosphate buffer, pH 7.4) containing 3 nM [³H]mepyramine. The nonspecific binding was determined in the presence of 10 μM cetirizine. The incubations were stopped by rapid dilution with ice-cold binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filters (Whatman, Vel, Belgium) that had been treated with 0.1% polyethylenimine. Filters were washed four times with binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting.

Molecular Modeling. Our H₁R homology model was obtained using the bovine rhodopsin crystal structure (Protein Data Bank entry 1L9H; Okada et al., 2002) as the template. Side chains were added using the SCWRL program (Canutescu et al., 2003). Water molecules present in the rhodopsin structure were retained, and their heavy atoms were kept fixed during all minimizations and molecular dynamic runs. The position of TM3 was manually changed with regard to the rhodopsin structure to avoid a clash between the

top of TM3 and TM2 (Lopez-Rodriguez et al., 2002). Given the presence of the H₁R-specific Trp¹⁵⁸ (4.56), TM3 could not be put into the position as found by molecular modeling studies on the 5HT_{1A} receptor. Therefore, we assumed an intermediate position between the location in the crystal structure of rhodopsin and the proposed location in the 5HT_{1A} receptor model. Short minimization runs were performed (5000 iterations using steepest descent) to refine the initial model. All minimizations were carried out while fixing the C α atoms to their initial positions.

Ligands were docked in the wild-type receptor using the automated docking procedure GOLD version 2.1 (Jones et al., 1997) applying default parameters. The complex of the A84S mutant receptor with the ligand was obtained by changing the appropriate residue in the WT receptor-ligand model to its guinea pig homolog. The obtained ligand-receptor complexes were used as input structures for further minimization and molecular dynamic studies. First, the position of the ligand is optimized by fixing the receptor except for the residues involved in ligand binding. Restraints were gradually released before final submission of the resulting complex to two simulated annealing runs at 600 K, each followed by cooling to 200 K before final minimization. In the first round of the simulated annealing run (2500-step initialization, 5000-step production, 5000-step cooling), the C α atoms of the receptor are fixed to their position as is the ligand. In the second round (15,000-step production, 5000-step cooling), the ligand is released and free to move. All minimizations and molecular dynamics simulations were performed using Discover (Accelrys, San Diego, CA).

Analytical Methods. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as a standard. Binding data were evaluated by a nonlinear, least-squares curve-fitting procedure using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Obtained pK_i, pEC₅₀, and K_d values are expressed as mean \pm S.E.M. of at least three independent experiments. Statistical analyses were carried out by nonpaired Student's *t* test. *P* values <0.05 were considered to indicate a significant difference (*P* < 0.05, *P* < 0.01, and *P* < 0.001). Despite significance, differences in pK_i values are only considered relevant when the difference is at least 0.3 logunits.

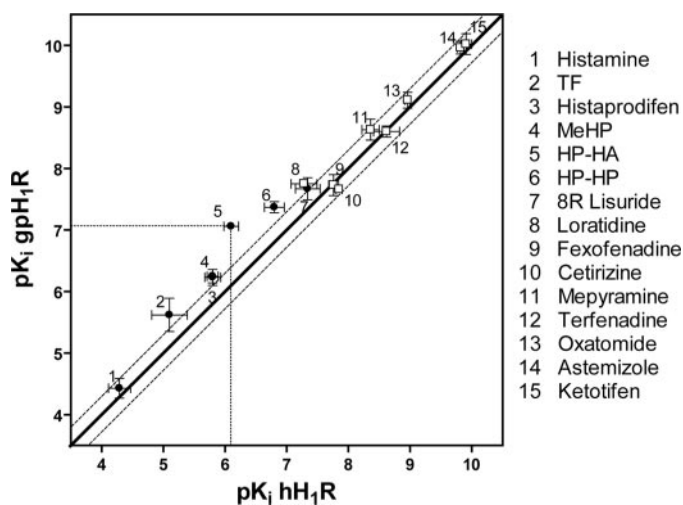


Fig. 1. Binding affinities (pK_i) of a variety of histaminergic agonists (closed symbols) and antagonists (open symbols) for the human and guinea pig H₁Rs. TF, 2-(3(trifluoromethylphenyl)histamine). The pK_i values for both wild-type human and guinea pig H₁Rs were determined by [³H]mepyramine displacements. pK_i values for HP-HA (5) are indicated by lines perpendicular to the x- and y-axes. A line with a slope of unity depicts the ideal correlation between the pK_i values for human and guinea pig H₁Rs; the dashed lines indicate 0.3 logunits deviation from unity. All values are calculated as mean \pm S.E.M. of at least three experiments.

Results and Discussion

Evaluation of Species Selectivity of H₁R Ligands.

Using displacement of [³H]mepyramine binding to guinea pig or human H₁Rs transiently expressed in COS-7 cells, we determined the affinity of a series of H₁R antagonists [cetirizine (Zyrtec), ketotifen (Zaditor), loratadine (Claritin), oxatomide (Tinset), fexofenadine (Allegra), astemizole, terfenadine, and mepyramine]. As shown in Fig. 1, none of the tested H₁R antagonists (open symbols) showed any preference, i.e., a difference in pK_i exceeding 0.3 log units (dotted lines), for binding to the guinea pig H₁R over the human H₁R, corroborating recent findings by Seifert et al. (2003). We also determined the binding affinities of several H₁R agonists (closed symbols) for both human and guinea pig H₁Rs. Again, the general rank order of affinities is shared between human and guinea pig H₁Rs, with histamine having the lowest and the recently characterized partial agonist 8R-lisuride (Bakker et al., 2004) having the highest H₁R affinity. Considering all tested agonists and antagonists, we observed a linear

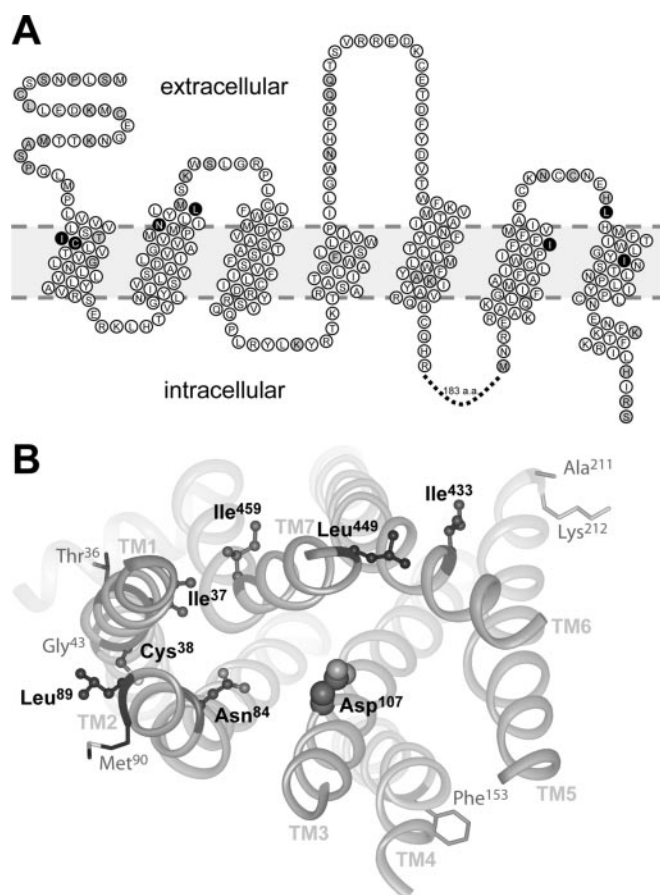


Fig. 2. Snake plot (A) and top view (B) of the human H₁R showing the topology of the TM helices. A, amino acids that are conserved in both guinea pig and human H₁Rs are depicted in white. Residues indicated in gray and black differ between human and guinea pig H₁Rs. The third intracellular loop is largely omitted from this graph (as indicated by 183 amino acids) because sequence homology in this region is very low. The residues in black have been selected (see text) in this study for a mutagenesis approach. B, the conserved Asp¹⁰⁷ (3.32) is shown space-filling for orientation. Side chains of amino acids that are conserved in both guinea pig and human H₁Rs are not shown in this view. Amino acids of which side chains are depicted differ between human and guinea pig H₁Rs. Amino acids selected for mutagenesis are shown as balls and sticks.

correlation ($r^2 = 0.96$) between human and guinea pig H_1R affinities over an affinity range of almost six decades. No species differences were observed between the human and the guinea pig H_1R for the affinities of the endogenous ligand histamine or the synthetic agonists histaprodifen, 2-(3-trifluoromethylphenyl)histamine, and 8*R*-lisuride. In contrast, MeHP exhibits a 3-fold higher affinity for the guinea pig H_1R than for the human H_1R . For the dimeric compounds HP-HP and HP-HA, the guinea pig over human H_1R -selectivity is even greater (4- and 10-fold, respectively). The higher affinities of these compounds for the guinea pig H_1R are in good agreement with the higher potencies of these agonists for guinea pig versus human H_1R s as recently demonstrated using a GTPase assay (Seifert et al., 2003).

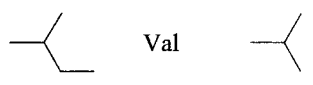
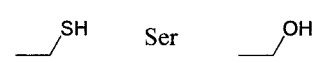
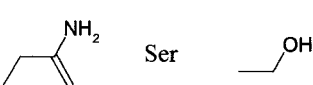
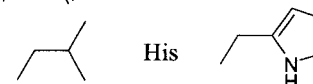
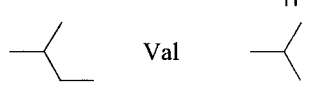
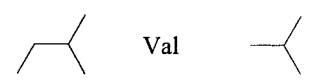

The species-dependent pharmacology of several of the histaprodifen analogs is also observed in functional assays. Measuring the effects on the contraction of the guinea pig ileum, HP-HA is up to 50-fold more potent than histamine (Christophe et al., 2003; Seifert et al., 2003), whereas in assays using heterologously expressed hH_1R s, both HP-HA and histamine seem to be equipotent (Seifert et al., 2003; Bruysters et al., 2004).

Generation and Evaluation of Human H_1R Mutants. The ligand-binding pocket of aminergic receptors is generally

considered to reside within the TM domains (Shi and Javitch, 2002). Within these TM domains, several amino acids have been identified in the human and guinea pig H_1R that are important for the interaction of ligands with the H_1R : Asp¹⁰⁷ (3.32) in TM3 (Ohta et al., 1994; Moguilevsky et al., 1998; Nonaka et al., 1998; Bruysters et al., 2004), Trp¹⁵⁸ (4.56) in TM4 (Wieland et al., 1999), Lys¹⁹¹ (5.39) and Asn¹⁹⁸ (5.46) in TM5 (Leurs et al., 1994, 1995; Ohta et al., 1994; Moguilevsky et al., 1995; Moguilevsky et al., 1998; Bruysters et al., 2004), and Phe⁴³² (6.52) and Phe⁴³⁵ (6.55) in TM6. None of these amino acids differ between human and guinea pig H_1R s. Actually, the sequence similarity within these TMs is high (93%), and only 12 amino acids differ between the two proteins (Fig. 2A). In the hH_1R , of these 12 amino acids, only Ile³⁷ (1.42) and Cys³⁸ (1.43) in TM1, Asn⁸⁴ (2.61) and Leu⁸⁹ (2.66) in TM2, and Leu⁴⁴⁹ (7.34) and Ile⁴⁵⁹ (7.44) in TM7 are predicted to be located either in proximity to the hydrophilic cleft in the hH_1R or on the interface of two TMs (Fig. 2B). We therefore reasoned that especially these amino acids may be directly involved in ligand binding and that one of these residues might be responsible for the observed differences in pharmacology between human and guinea pig H_1R s. To test this hypothesis, we created the following mutant hH_1R s in which the selected amino acids are individually replaced into

TABLE 1

Affinities of [³H]mepyramine and expression levels of human and guinea pig H_1R s and several human H_1R mutants. The values are determined by saturation [³H]mepyramine binding assays. Data were calculated as the mean \pm S.E.M. for at least three experiments, each performed in duplicate. Positions of mutations are depicted using the Ballesteros and Weinstein index (Ballesteros and Weinstein, 1995).

	[³ H]Mepyramine		Location and Nature of Mutations			
	<i>K_d</i>	<i>B_{max}</i>	Position	a.a. hH_1R	a.a. gpH_1R	
	<i>nM</i>	<i>pmol/mg</i>				
hH_1R	1.2 \pm 0.3	12.1 \pm 0.9				
gpH_1R	0.5 \pm 0.1	5.3 \pm 0.8 ^a				
hH_1R -Ile ³⁷ Val	1.7 \pm 0.5	11.4 \pm 1.5	1.42	Ile	Val	
hH_1R -Cys ³⁸ Ser	1.4 \pm 0.1	17.1 \pm 2.1	1.43	Cys	Ser	
hH_1R -Asn ⁸⁴ Ser	1.1 \pm 0.3	6.5 \pm 1.2 ^b	2.61	Asn	Ser	
hH_1R -Leu ⁸⁹ His	>30	N.D.	2.66	Leu	His	
hH_1R -Ile ⁴³³ Val	0.7 \pm 0.1	9.9 \pm 2.8	6.53	Ile	Val	
hH_1R -Leu ⁴⁴⁹ Val	1.4 \pm 0.2	9.7 \pm 2.7	7.34	Leu	Val	
hH_1R -Ile ⁴⁵⁹ Leu	1.0 \pm 0.2	10.3 \pm 1.5	7.44	Ile	Leu	

a.a., amino acid; N.D., not determined.

^a $P < 0.01$.

^b $P < 0.05$.

their guinea pig counterparts: hH₁R Ile³⁷Val, hH₁R Cys³⁸Ser, hH₁R Asn⁸⁴Ser, hH₁R Leu⁸⁹His, hH₁R Leu⁴⁴⁹Val, and hH₁R Ile⁴⁵⁹Leu. Although in our H₁R model Ile⁴³³ (6.53) points toward the plasma membrane, we also included the mutant hH₁R Ile⁴³³Val receptor in our study because Ile⁴³³ is located in between the established hH₁R-ligand interaction points Phe⁴³² (6.52) and Phe⁴³⁵ (6.55). In general, we noticed that, at the selected positions, the amino acids present in the

human H₁R are bulkier than their guinea pig H₁R counterparts (Table 1).

Most of the generated mutant H₁Rs are expressed at comparable levels (B_{\max} values of ~ 10 pmol/mg) and bind [³H]mepyramine with unchanged affinity (K_d values of 0.5–1.7 nM) compared with wild-type human H₁Rs (Table 1). However, the mutant receptor hH₁R-Leu⁸⁹His (2.66), with a point mutation in the top of TM2, did not show any

TABLE 2

Binding affinities (pK_i) of H₁R agonists for human and guinea pig H₁Rs and several human H₁R mutants

The pK_i values are determined by [³H]mepyramine displacements. Data were calculated as the mean \pm S.E.M. of at least three experiments.

	Cetirizine	Histamine	HP-HA	HP-HP	VUF 4669	VUF 8401
hH ₁ R	7.9 \pm 0.1	4.3 \pm 0.2	6.1 \pm 0.1	6.8 \pm 0.2	7.7 \pm 0.1	6.0 \pm 0.1
gpH ₁ R	7.7 \pm 0.1 ^a	4.4 \pm 0.2	7.1 \pm 0.1 ^b	7.4 \pm 0.1 ^a	9.0 \pm 0.1 ^b	6.9 \pm 0.1 ^b
hH ₁ R-Ile ³⁷ Val	7.7 \pm 0.1	3.9 \pm 0.2	5.9 \pm 0.1	6.9 \pm 0.1	8.0 \pm 0.1	5.9 \pm 0.1
hH ₁ R-Cys ³⁸ Ser	7.9 \pm 0.1	3.9 \pm 0.1	6.1 \pm 0.1	6.8 \pm 0.1	8.0 \pm 0.1	6.0 \pm 0.1
hH ₁ R-Asn ⁸⁴ Ser	7.5 \pm 0.1 ^b	4.2 \pm 0.1	6.8 \pm 0.1 ^b	7.3 \pm 0.1 ^a	8.9 \pm 0.1 ^b	6.4 \pm 0.1 ^a
hH ₁ R-Ile ⁴³³ Val	8.0 \pm 0.1	4.5 \pm 0.2	6.2 \pm 0.1	7.1 \pm 0.1	8.0 \pm 0.1	6.1 \pm 0.1
hH ₁ R-Leu ⁴⁴⁹ Val	7.9 \pm 0.1	4.0 \pm 0.1	6.0 \pm 0.1	7.0 \pm 0.1	8.1 \pm 0.1	6.0 \pm 0.1
hH ₁ R-Ile ⁴⁵⁹ Leu	8.0 \pm 0.1	4.4 \pm 0.1	6.0 \pm 0.1	7.0 \pm 0.1	7.8 \pm 0.2	6.0 \pm 0.1

^a $P < 0.05$.

^b $P < 0.01$ versus hH₁R.

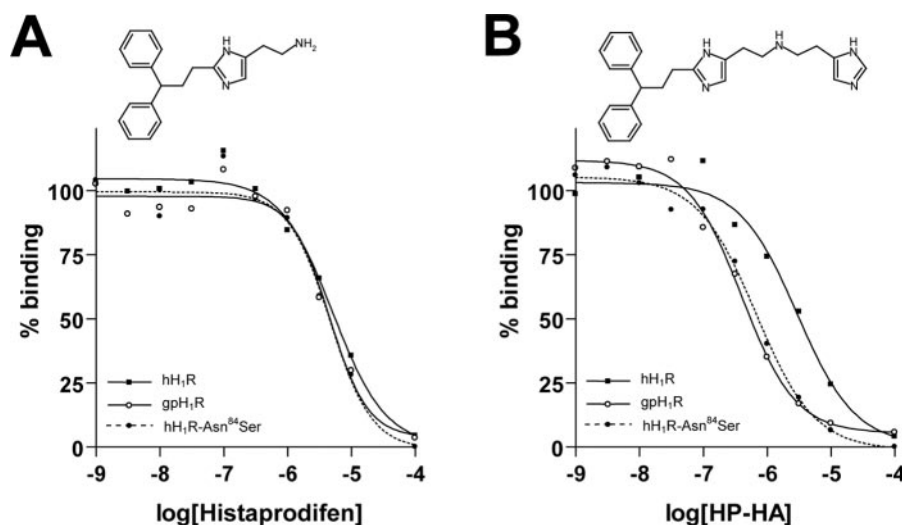


Fig. 3. Displacement of [³H]mepyramine binding to wild-type hH₁Rs (■) and gpH₁Rs (○) and to the mutant receptor hH₁R Asn⁸⁴Ser (●) by histaprodifen (A) and the HP-HA dimer (B). A representative experiment is shown.

[³H]mepyramine binding at concentrations up to 30 nM and may not be properly expressed at the cell membrane. Displacement of [³H]mepyramine binding indicated that all tested mutant H₁Rs bind the endogenous agonist histamine with unchanged affinity (Table 2). Only for mutant hH₁R Asn⁸⁴Ser (2.61) receptors, which harbor a point mutation in TM2, the affinities for HP-HA are increased ($pK_i = 6.8$) compared with the wild-type hH₁R ($pK_i = 6.1$), resulting in a gpH₁R-like ($pK_i = 7.1$) pharmacology (Fig. 3; Table 2). In addition, for HP-HP, the species difference was reversed by the Asn⁸⁴Ser mutation (Table 2).

HP-HA is an agonist for the hH₁R as measured using a $G_{\alpha_{11}}$ -mediated nuclear factor- κ B reporter gene assay ($pEC_{50} = 6.4 \pm 0.1$) with a potency comparable with histamine ($pEC_{50} = 6.4 \pm 0.2$) (Bruysters et al., 2004). For both the gpH₁R and mutant hH₁R Asn⁸⁴Ser (2.61), the potency of HP-HA (pEC_{50} values of 7.2 ± 0.1 and 7.9 ± 0.1 , respectively) strongly exceeds that of histamine (pEC_{50} values of 6.0 ± 0.1 and 6.5 ± 0.1 , respectively). These findings confirm that also in a functional assay we observe species-specific H₁R pharmacology, and the mutant hH₁R Asn⁸⁴Ser not only displays a guinea pig H₁R binding profile but also a guinea pig H₁R functional profile.

These data suggest that residue Asn/Ser⁸⁴ (2.61) is of critical importance for the observed species-dependent agonist

pharmacology of the human and guinea pig H₁Rs. Moreover, these data also indicate that for some H₁R agonists TM2 is part of the H₁R ligand binding-pocket. Interestingly, both human and rat H₁Rs have an asparagine at position 2.61. Measuring endothelium-dependent relaxation of rat aortic rings, Menghin et al. (2003) have shown that MeHP and HP-HA are equipotent, corroborating our previous findings with human H₁Rs expressed in COS-7 cells (Bruysters et al., 2004). However, measuring guinea pig ileum contractions, the potency of HP-HA exceeds that of MeHP 10-fold (Menghin et al., 2003). These observations further strengthen the involvement of Asn/Ser⁸⁴ (2.61) in the species-dependent H₁R pharmacology. Consequently, pharmacological observations with rat H₁Rs will have more predictive power for the action of ligands at human H₁Rs.

Characterization of a Novel, Species-Selective H₁R Antagonist. The H₁R species-selective interactions were originally observed for bulky H₁R agonists (HP-HA and HP-HP). These compounds seem to interact with the “classical” binding pocket (TMs 3, 4, 5, and 6) and Asn/Ser⁸⁴ (2.61), hereby defining an additional binding pocket near TM2. To test whether the additional interactions are restricted to agonists alone, or are also possible for antagonists, we screened an in-house library of H₁R antagonists. From this selection, VUF 4669 was identified as an H₁R antagonist,

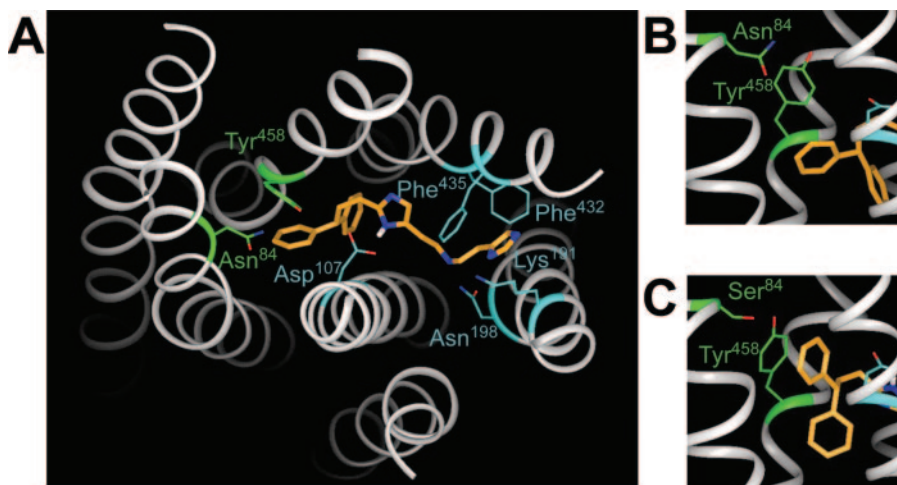


Fig. 4. Binding models of HP-HA (orange) in the human H₁R. View from the extracellular side (A) of the wild-type hH₁R. Detailed snapshot of view parallel to TM domains of wild-type (B) and mutant hH₁R Asn⁸⁴Ser (C). Amino acids previously known to interact with H₁R ligands are depicted in blue [Asp¹⁰⁷ (3.32), Lys¹⁹¹ (5.39), Asn¹⁹⁸ (5.46), Phe⁴³² (6.52) and Phe⁴³⁵ (6.55)], and Asn/Ser⁸⁴ (2.61) and Tyr⁴⁵⁸ (7.43) are depicted in green.

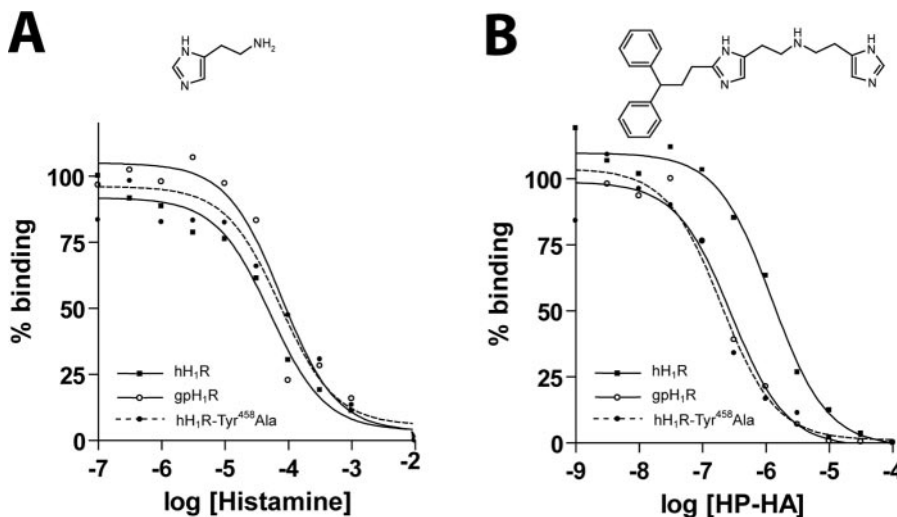


Fig. 5. Displacement of [³H]mepyramine binding to wild-type hH₁Rs (■) and gpH₁Rs (○) and to the mutant receptor hH₁R Tyr⁴⁵⁸Ala (●) by histamine (A) and HP-HA dimer (B). A representative experiment is shown.

which differentiates significantly between human and guinea pig H₁Rs. VUF 4669 showed a 17-fold increase in binding affinity for the guinea pig H₁R ($pK_i = 9.0 \pm 0.1$), compared with its affinity for the human H₁R ($pK_i = 7.7 \pm 0.1$) (Table 2). Apparently, the concept of species-selective binding is not restricted to H₁R agonists but can also be observed for certain H₁R antagonists. Again, VUF 4669 exhibits an increased affinity for the mutant hH₁ Asn⁸⁴Ser receptor ($pK_i = 8.9 \pm 0.1$), confirming the guinea pig-like pharmacological profile of this mutant human H₁R. The other human to guinea pig H₁R mutants used in this study exhibit an affinity for VUF 4669 that is identical to the affinity for the WT human H₁R (Table 2).

Previously, also several arpromidine analogs, which display both H₁R antagonistic and H₂R agonistic properties, were characterized as guinea pig H₁R-preferring compounds (Seifert et al., 2003). Indeed, VUF 8401, a structural analog of arpromidine displays a 9-fold higher affinity for the guinea pig H₁R than for the human H₁R (Table 2). In addition, VUF 8401 binds with an increased affinity to the mutant hH₁R Asn⁸⁴Ser (2.61) (Table 2), although this mutation did not fully reverse the species difference. None of the other mutant hH₁Rs show an increased affinity for VUF 8401 (Table 2). Interaction with Asn/Ser⁸⁴ (2.61) therefore partially explains the observed species difference. We hypothesize that for arpromidine-like ligands the higher affinity depends on Ser⁸⁴ (2.61) and additional guinea pig H₁R-specific residues. This hypothesis will be the basis of future investigations.

Like HP-HA and VUF 4669, arpromidine analogs are bulky ligands, having aromatic moieties on either side of a protonated moiety, and we hypothesize that these features are mandatory for species selectivity. H₁R antagonists such as terfenadine, fexofenadine, and oxatamide, however, also show such features, but they seem not to be species-selective. Clearly, the simple presence of two aromatic domains in a ligand is not the only denominator for species selectivity.

Rationalization of the Role of Asn⁸⁴ (2.61) in the Species-Selective Binding. To rationalize the potential role of the amino acid at position 2.61 (Asn/Ser) in the species-selective binding of HP-HA, we created a homology model for the human H₁R on the basis of the available structural information on bovine rhodopsin (Palczewski et al., 2000; Okada et al., 2002). In the absence of ligand, our H₁R homology model suggests hydrogen bonding between Asn⁸⁴ (2.61) and Tyr⁴⁵⁸ (7.43), a residue that is conserved between human and guinea pig H₁Rs. Using the automated docking procedure GOLD version 2.1 (Jones et al., 1997), we subsequently docked the agonist HP-HA in the receptor model (Fig. 4A). In contrast to H₁R antagonists such as cetirizine, the diphenyl moiety of HP-HA is not oriented toward TM6, but it is predicted to point toward TMs 1, 2, and 7, confirming our previous suggestions based on site-directed mutagenesis studies of the histamine binding pocket (Bruysters et al., 2004). Thereafter, we changed Asn⁸⁴ (2.61) into Ser, thus creating a model of the hH₁R Asn⁸⁴Ser receptor containing HP-HA (Asn⁸⁴Ser model). Molecular dynamics simulations were subsequently performed to optimize both HP-HA containing WT and Asn⁸⁴Ser models. During both simulations, hydrogen bonding was maintained between Asn⁸⁴ (2.61) and Tyr⁴⁵⁸ (7.43) in the WT model (3.31 Å; Fig. 4B) and between Ser⁸⁴ (2.61) and Tyr⁴⁵⁸ (7.43) in the Asn⁸⁴Ser model (2.80 Å; Fig. 4C). However, the orientation of Tyr⁴⁵⁸ differs between

both models, probably because of the structural differences between Ser and Asn at position 2.61 (e.g., length and flexibility of the side chain). Because the affinity of HP-HA is higher for the Asn⁸⁴Ser H₁R, the conformation of HP-HA in the Asn⁸⁴Ser model is considered more favorable. In the WT model, Tyr⁴⁵⁸ occupies the space that in the Asn⁸⁴Ser model is occupied by one of the phenyl rings of HP-HA. Our computational studies therefore suggest that Tyr⁴⁵⁸ might sterically hinder the binding of HP-HA in the hH₁R, thereby "forcing" HP-HA to bind deeper within the receptor.

To test the potential involvement of Tyr⁴⁵⁸ (7.43) in the binding of HP-HA to the human H₁R, we mutated Tyr⁴⁵⁸ (7.43) in the human H₁R into an alanine (hH₁R Tyr⁴⁵⁸Ala). Saturation binding analysis using [³H]mepyramine shows that this mutant H₁R is expressed at comparable levels ($B_{\max} = 8.2 \pm 3.5$ pmol/mg protein) and with an unchanged affinity for [³H]mepyramine ($K_d = 3.0 \pm 0.7$) in comparison with the wild-type H₁R. The Tyr⁴⁵⁸Ala mutation did also not affect the affinity for histamine ($pK_i = 4.4 \pm 0.2$) (Fig. 5). Because the mutation Tyr⁴⁵⁸Ala would remove potential steric hindrance between HP-HA and the hH₁R, we expected an increased affinity of HP-HA. Indeed, mutation of Tyr⁴⁵⁸ into an alanine results in a 5-fold increase in affinity for HP-HA ($pK_i = 6.8 \pm 0.1$) compared with the wild-type H₁R (Fig. 5). This affinity is similar to the affinity of HP-HA for both the gpH₁R ($pK_i = 7.1 \pm 0.1$) and the hH₁R Asn⁸⁴Ser ($pK_i = 6.8 \pm 0.1$) (Table 2).

The results of our computational and mutagenesis studies indicate an important role of Asn⁸⁴ (2.61) as selectivity switch. Moreover, our results illustrate the first structural features of an additional binding pocket between TM2 and TM7 in the H₁R. Residues in both TM2 and TM7 have been implicated in ligand binding for only a few other aminergic receptors (for review, see Shi and Javitch, 2002). For example, bulky H₂R agonists were suggested to interact with TM7 in the H₂ receptor (Kelley et al., 2001), whereas dopamine D₂/D₄ receptor subtype selectivity of several classes of antagonists is determined by a hydrophobic microdomain formed by six amino acids in TM2, TM3, and TM7 (including position 2.61) (Javitch et al., 1999). Also for adrenergic receptors, the key to β_1/β_2 agonist selectivity seems to be localized in TMs 2 and 7 (Isogaya et al., 1999). Moreover, amino acids present at position 7.43 (homologous to hH₁R Tyr⁴⁵⁸) are demonstrated to be involved in ligand binding to 5HT_{2A} (Roth et al., 1997) and muscarinic acetylcholine M₃ receptors (Wess et al., 1991). The involvement of TMs 2 and 7 in the H₁R binding pocket of some H₁R ligands is therefore highly likely.

Conclusions

In conclusion, the human and guinea pig H₁Rs exhibit significantly different affinities for agonists, such as HP-HA and HP-HP, as well as for several antagonists such as VUF 4669 and VUF 8401. These differences can be explained by the presence of Asn⁸⁴ (2.61) in the hH₁R versus Ser⁸⁴ (2.61) in the gpH₁R. Based on molecular dynamics simulations and site-directed mutagenesis data, we suggest a possible role for Tyr⁴⁵⁸ (7.43) in the binding of certain H₁R ligands. Our data provide the first evidence that for these H₁R ligands, TM2 and TM7 are also part of the ligand binding pocket. Exploitation of these additional interaction points within the H₁R ligand binding pocket in drug development programs may

yield a new generation of antihistamines with increased structural diversity compared with the currently known ligands.

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References

- Bakker RA, Schoonus SB, Smit MJ, Timmerman H, and Leurs R (2001) Histamine H₁-receptor activation of nuclear factor- κ B: roles for G $\beta\gamma$ - and G $\alpha_{q/11}$ -subunits in constitutive and agonist-mediated signaling. *Mol Pharmacol* **60**:1133–1142.
- Bakker RA, Weiner DM, ter Laak T, Beuming T, Zuiderveld OP, Edelbroek M, Hacksell U, Timmerman H, Brann MR, and Leurs R (2004) 8R-lisuride is a potent stereospecific histamine H₁-receptor partial agonist. *Mol Pharmacol* **65**:538–549.
- Ballesteros J and Weinstein H (1995) Integrated methods for the construction of three-dimensional models of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25**:366–428.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254.
- Bruysters M, Pertz HH, Teunissen A, Bakker RA, Gillard M, Chatelain P, Schunack W, Timmerman H, and Leurs R (2004) Mutational analysis of the histamine H₁-receptor binding pocket of histaprodifens. *Eur J Pharmacol* **487**:55–63.
- Canutescu AA, Shelenkov AA, and Dunbrack RL Jr (2003) A graph-theory algorithm for rapid protein side-chain prediction. *Protein Sci* **12**:2001–2014.
- Christophe B, Carlier B, Schunack W, Chatelain P, Peck MJ, and Massingham R (2003) Histamine H₁-agonist properties of histaprodifen derivatives on guinea-pig isolated trachea and ileum. *Inflamm Res* **52**:S51–S52.
- Elz S, Kramer K, Pertz HH, Detert H, ter Laak AM, Kuhne R, and Schunack W (2000) Histaprodifens: synthesis, pharmacological in vitro evaluation and molecular modeling of a new class of highly active and selective histamine H₁-receptor agonists. *J Med Chem* **43**:1071–1084.
- Fukui H, Fujimoto K, Mizuguchi H, Sakamoto K, Horio Y, Takai S, Yamada K, and Ito S (1994) Molecular cloning of the human histamine H₁ receptor gene. *Biochem Biophys Res Commun* **201**:894–901.
- Gillard M, Van Der Perren C, Moguilevsky N, Massingham R, and Chatelain P (2002) Binding characteristics of cetirizine and levocetirizine to human H₁ histamine receptors: contribution of Lys¹⁹¹ and Thr¹⁹⁴. *Mol Pharmacol* **61**:391–399.
- Goldman LA, Cutrone EC, Kutenko SV, Krause CD, and Langer JA (1996) Modifications of vectors pEF-BOS, pcDNA1 and pcDNA3 result in improved convenience and expression. *Biotechniques* **21**:1013–1015.
- Hill SJ, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R, and Haas HL (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* **49**:253–278.
- Isogaya M, Sugimoto Y, Tanimura R, Tanaka R, Kikkawa H, Nagao T, and Kurose H (1999) Binding pockets of the β_1 - and β_2 -adrenergic receptors for subtype-selective agonists. *Mol Pharmacol* **56**:875–885.
- Javitch JA, Ballesteros JA, Chen J, Chiappa V, and Simpson MM (1999) Electrostatic and aromatic microdomains within the binding-site crevice of the D2 receptor: contributions of the second membrane-spanning segment. *Biochemistry* **38**:7961–7968.
- Jones G, Willett P, Glen RC, Leach AR, and Taylor R (1997) Development and validation of a genetic algorithm for flexible docking. *J Mol Biol* **267**:727–748.
- Kelley MT, Burckstummer T, Wenzel-Seifert K, Dove S, Buschauer A, and Seifert R (2001) Distinct interaction of human and guinea pig histamine H₂-receptor with guanidine-type agonists. *Mol Pharmacol* **60**:1210–1225.
- Leschke C, Elz S, Garbarg M, and Schunack W (1995) Synthesis and histamine H₁ receptor agonist activity of a series of 2-phenylhistamines, 2-heteroarylhistamines and analogues. *J Med Chem* **38**:1287–1294.
- Leurs R, Smit MJ, Meeder R, Ter Laak AM, and Timmerman H (1995) Lysine²⁰⁰ located in the fifth transmembrane domain of the histamine H₁ receptor interacts with histamine but not with all H₁ agonists. *Biochem Biophys Res Commun* **214**:110–117.
- Leurs R, Smit MJ, Tensen CP, Ter Laak AM, and Timmerman H (1994) Site-directed mutagenesis of the histamine H₁-receptor reveals a selective interaction of asparagine²⁰⁷ with subclasses of H₁-receptor agonists. *Biochem Biophys Res Commun* **201**:295–301.
- Lopez-Rodriguez ML, Vicente B, Deupi X, Barrondo S, Olivella M, Morcillo MJ, Behamu B, Ballesteros JA, Salles J, and Pardo L (2002) Design, synthesis and pharmacological evaluation of 5-hydroxytryptamine(1a) receptor ligands to explore the three-dimensional structure of the receptor. *Mol Pharmacol* **62**:15–21.
- Menghin S, Pertz HH, Kramer K, Seifert R, Schunack W, and Elz S (2003) N⁶-Imidazolylalkyl and pyridylalkyl derivatives of histaprodifen: synthesis and in vitro evaluation of highly potent histamine H₁-receptor agonists. *J Med Chem* **46**:5458–5470.
- Moguilevsky N, Differding E, Gillard M, and Bollen A (1998) Rational drug design using mammalian cell lines expressing site-directed mutants of the human H₁ histaminic receptor. *Anim Cell Technol: Basic Appl Asp* **9**:65–69.
- Moguilevsky N, Varsalona F, Guillaume JP, Noyer M, Gillard M, Daliers J, Henichart JP, and Bollen A (1995) Pharmacological and functional characterisation of the wild-type and site-directed mutants of the human H₁ histamine receptor stably expressed in CHO cells. *J Recept Signal Transduct Res* **15**:91–102.
- Nonaka H, Otaki S, Ohshima E, Kono M, Kase H, Ohta K, Fukui H, and Ichimura M (1998) Unique binding pocket for KW-4679 in the histamine H₁ receptor. *Eur J Pharmacol* **345**:111–117.
- Ohta K, Hayashi H, Mizuguchi H, Kagamiyama H, Fujimoto K, and Fukui H (1994) Site-directed mutagenesis of the histamine H₁ receptor: roles of aspartic acid¹⁰⁷, asparagine¹⁹⁸ and threonine¹⁹⁴. *Biochem Biophys Res Commun* **203**:1096–1101.
- Okada T, Fujiyoshi Y, Silow M, Navarro J, Landau EM, and Shichida Y (2002) Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proc Natl Acad Sci USA* **99**:5982–5987.
- Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science (Wash DC)* **289**:739–745.
- Roth BL, Shoham M, Choudhary MS, and Khan N (1997) Identification of conserved aromatic residues essential for agonist binding and second messenger production at 5-hydroxytryptamine_{2A} receptors. *Mol Pharmacol* **52**:259–266.
- Seifert R, Wenzel-Seifert K, Burckstummer T, Pertz HH, Schunack W, Dove S, Buschauer A, and Elz S (2003) Multiple differences in agonist and antagonist pharmacology between human and guinea pig histamine H₁-receptor. *J Pharmacol Exp Ther* **305**:1104–1115.
- Shi L and Javitch JA (2002) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol* **42**:437–467.
- ter Laak AM, Venhorst J, Donne-Op den Kelder GM, and Timmerman H (1995) The histamine H₁-receptor antagonist binding site. A stereoselective pharmacophoric model based upon (semi-)rigid H₁-antagonists and including a known interaction site on the receptor. *J Med Chem* **38**:3351–3360.
- Wess J, Gdula D, and Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. *EMBO (Eur Mol Biol Organ) J* **10**:3729–3734.
- Wieland K, Laak AM, Smit MJ, Kuhne R, Timmerman H, and Leurs R (1999) Mutational analysis of the antagonist-binding site of the histamine H₁ receptor. *J Biol Chem* **274**:29994–30000.
- Zhang M-Q, Leurs R, and Timmerman H (1997) Histamine H₁-receptor antagonists, in *Burger's Medicinal Chemistry and Drug Discovery* (Wolf ME ed) pp 495–559, John Wiley & Sons, Inc., New York.
- Zingel V, Leschke C, and Schunack W (1995) Developments in histamine H₁-receptor agonists. *Prog Drug Res* **44**:49–85.

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