0026-895X/05/6704-1045–1052$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
*Mol Pharmacol* 67:1045–1052, 2005

Vol. 67, No. 4
8847/1197180
*Printed in U.S.A.*



# Pharmacological Differences between Human and Guinea Pig Histamine $H_1$ Receptors: $Asn^{84}$ (2.61) as Key Residue within an Additional Binding Pocket in the $H_1$ Receptor

Martijn Bruysters, Aldo Jongejan, Michel Gillard, Frank van de Manakker, Remko A. Bakker, Pierre Chatelain, and Rob Leurs

*Leiden/Amsterdam Center for Drug Research, Faculty of Sciences, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands (M.B., A.J., F.v.d.M., R.A.B., R.L.); and UCB S.A. Pharma Sector, Braine-l'Alleud, Belgium (M.G., P.C.)*

Received October 29, 2004; accepted December 29, 2004

## ABSTRACT

We tested several histamine $H_1$ receptor ($H_1R$) agonists and antagonists for their differences in binding affinities between human and guinea pig $H_1Rs$ 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 $H_1Rs$. 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 $H_1R$ 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 $H_1Rs$ in which amino acids were individually replaced by their guinea pig $H_1R$ counterparts. Residue $Asn^{84}$ (2.61) in transmembrane domain (TM) 2 seemed to act as a selectivity switch in the $H_1R$. Molecular modeling and site-directed mutagenesis studies suggest that $Asn^{84}$ interacts with the conserved $Tyr^{458}$ (7.43) in TM7. Our data provide the first evidence that for some $H_1R$ 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 $H_1$, $H_2$, $H_3$, and $H_4$ receptors. The $H_1$ receptor ($H_1R$) regulates inflammatory and allergic responses and is successfully targeted by various drugs. $H_1R$ antagonists have been on the market since 1942 for the treatment of allergies, and newer, nonsedating second generation $H_1R$ antagonists are still the medication of choice to relief certain allergic symptoms (Hill et al., 1997).

In contrast to the development of various potent $H_1R$ antagonists, the synthesis of selective and potent $H_1R$ agonists has not achieved the same success (Hill et al., 1997). Only in 1995, 2-(3-trifluoromethylphenyl)histamine was discovered as the first selective $H_1R$ agonist with a potency equal to histamine as determined by the $H_1R$-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 $H_1R$ agonists, the histaprodifens. Histaprodifen combines a histamine moiety linked at the 2-position with an $\omega,\omega$-diphenylalkyl substituent, a characteristic of the $H_1R$ antagonist pharmacophore (ter Laak et al., 1995; Zhang et al., 1997). Based on this new $H_1R$ agonist, "dimeric" histaprodifens were subsequently developed, consisting of a histaprodifen moiety linked at the N$\alpha$ 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

This study was supported in part by UCB Pharma.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.008847.

**ABBREVIATIONS:** GPCR, G protein-coupled receptor; $H_1R$, histamine $H_1$ receptor; HP-HA, histaprodifen-histamine dimer; HP-HP, histaprodifen-histaprodifen dimer; MeHP, N$\alpha$-methylhistaprodifen; TM, transmembrane domain; $hH_1R$, human histamine $H_1$ 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; $gpH_1R$, guinea pig histamine $H_1$ receptor.

human $H_1$Rs 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 $H_1$R recognition of these $H_1$R agonists.

In aminergic GPCRs, including the $H_1$R, 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 $H_1$R $Asp^{107}$ in TM3 (residue 3.32 according to the Ballesteros-Weinstein numbering) is part of the binding pocket of both $H_1$R 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 $H_1$R binding pocket of histamine: $Lys^{191}$ (5.39) (Leurs et al., 1995; Moguilevsky et al., 1998; Wieland et al., 1999; Gillard et al., 2002; Bruysters et al., 2004), $Asn^{198}$ (5.46) (Leurs et al., 1994; Ohta et al., 1994; Moguilevsky et al., 1995; Bruysters et al., 2004), and $Phe^{435}$ (6.55) (Bruysters et al., 2004) are considered to accommodate the imidazole ring of histamine. The $H_1$R antagonist binding pocket stretches deeper into the receptor protein and comprises the aromatic amino acids $Trp^{158}$ (4.56) (Wieland et al., 1999) and $Phe^{432}$ (6.52) (Wieland et al., 1999; Bruysters et al., 2004).

Recently, we studied the binding pocket of several histaprodifen analogs in the human $H_1$R (Bruysters et al., 2004). We demonstrated that histamine and the histamine moiety of histaprodifens bind to the human $H_1$R in a similar orientation. Although the diphenylalkyl system of histaprodifen interacts with the $H_1$R in an "antagonistic binding mode", i.e., interacting with $Phe^{432}$ (6.52) in TM6 (Bruysters et al., 2004), no interactions with $Lys^{191}$ (5.39) and $Phe^{435}$ (6.55) were found. Again, the interaction with both $Asp^{107}$ (3.32) proved crucial. Although $Asn^{198}$ (5.46) did not affect histaprodifen affinity, it seemed pivotal for agonist-induced activation of the $hH_1$R. An interaction between $Asn^{198}$ (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 $H_1$Rs by a combined approach of molecular modeling and site-directed mutagenesis. We reevaluated several $H_1$R agonists and antagonists for their differences in affinity between human and guinea pig $H_1$Rs by [$^3$H]mepyramine displacement studies. Based on our knowledge of the $H_1$R binding site of the histaprodifens and the high (93%) level of sequence homology within the TM domains of the human and guinea pig $H_1$Rs, we extended our approach to mutant human $H_1$Rs in which selected amino acids were individually replaced by their guinea pig $H_1$R counterparts. Using this strategy, we identified $Asn^{84}$ (2.61) in TM2 as the molecular basis for the observed species selectivity of certain $H_1$R 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). Oxatomide 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*$\alpha$-methyl-histaprodifen dihydrogen oxalate), histaprodifen-histaprodifen dimer trihydrogenoxalate and histaprodifen-histamine dimer (*N*$\alpha$-[2-(1*H*-imidazol-4yl)-ethyl]-histaprodifen) trihydrogenoxalate) (Dr. W. Schunack, Free University, Berlin, Germany), the cDNA encoding the human $H_1$R (Dr. H. Fukui, University of Tokushima, Tokushima, Japan), and the expression vector $pcDEF_3$ (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). [$^3$H]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 $H_1$R.

**Site-Directed Mutagenesis.** The cDNA encoding the human $H_1$R (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 $pcDEF_3$ (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% $CO_2$, 95% air atmosphere in Dulbecco's modified Eagle's medium containing 50 IU/ml penicillin, 50 $\mu$g/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 $\mu$g 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.

**$H_1$R Binding Studies.** The COS-7 cell homogenates (~5 $\mu$g) were incubated for 60 min at 30°C in 500 $\mu$l of binding buffer (50 mM $Na_2$/K-phosphate buffer, pH 7.4) containing 3 nM [$^3$H]mepyramine. The nonspecific binding was determined in the presence of 10 $\mu$M 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 $H_1$R 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 $H_1R$-specific $Trp^{158}$ (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$\alpha$ 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$\alpha$ 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 p$K_i$, p$EC_{50}$, and $K_d$ values are expressed as mean ± 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 p$K_i$ values are only considered relevant when the difference is at least 0.3 logunits.

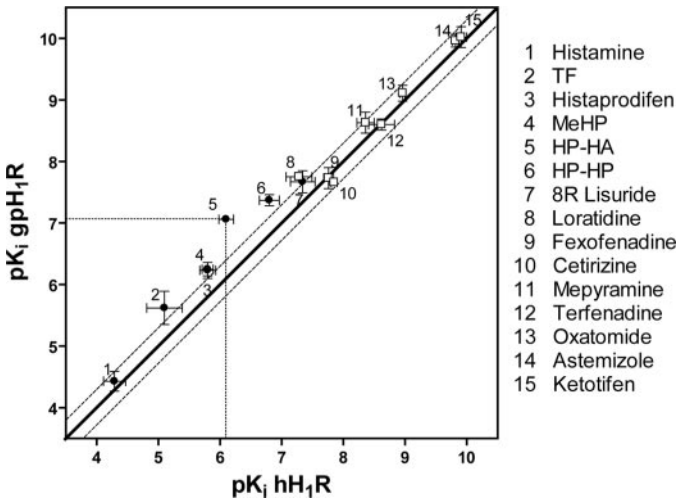



**Fig. 1.** Binding affinities (p$K_i$) of a variety of histaminergic agonists (closed symbols) and antagonists (open symbols) for the human and guinea pig $H_1Rs$. TF, 2-(3(trifluoremethylphenyl)histamine. The p$K_i$ values for both wild-type human and guinea pig $H_1Rs$ were determined by [$^3$H]mepyramine displacements. p$K_i$ 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 p$K_i$ values for human and guinea pig $H_1Rs$; the dashed lines indicate 0.3 logunits deviation from unity. All values are calculated as mean ± S.E.M. of at least three experiments.

## Results and Discussion

**Evaluation of Species Selectivity of $H_1R$ Ligands.** Using displacement of [$^3$H]mepyramine binding to guinea pig or human $H_1Rs$ transiently expressed in COS-7 cells, we determined the affinity of a series of $H_1R$ antagonists [cetirizine (Zyrtec), ketotifen (Zaditor), loratadine (Claritin), oxatomide (Tinset), fexofenadine (Allegra), astemizole, terfenadine, and mepyramine]. As shown in Fig. 1, none of the tested $H_1R$ antagonists (open symbols) showed any preference, i.e., a difference in p$K_i$ exceeding 0.3 log units (dotted lines), for binding to the guinea pig $H_1R$ over the human $H_1R$, corroborating recent findings by Seifert et al. (2003). We also determined the binding affinities of several $H_1R$ agonists (closed symbols) for both human and guinea pig $H_1Rs$. Again, the general rank order of affinities is shared between human and guinea pig $H_1Rs$, with histamine having the lowest and the recently characterized partial agonist 8$R$-lisuride (Bakker et al., 2004) having the highest $H_1R$ affinity. Considering all tested agonists and antagonists, we observed a linear

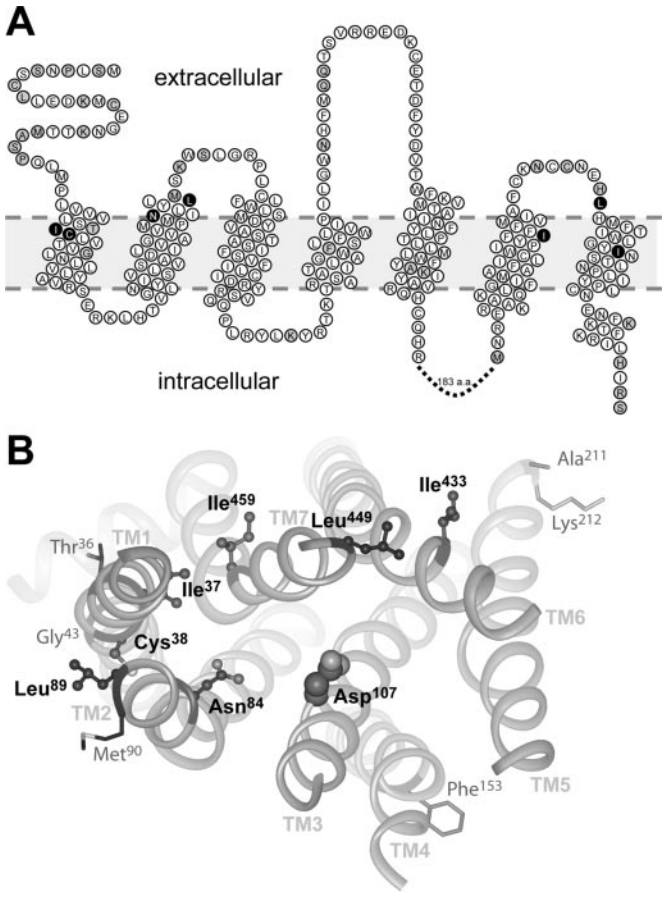



**Fig. 2.** Snake plot (A) and top view (B) of the human $H_1R$ showing the topology of the TM helices. A, amino acids that are conserved in both guinea pig and human $H_1Rs$ are depicted in white. Residues indicated in gray and black differ between human and guinea pig $H_1Rs$. The third intracellular 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 $Asp^{107}$ (3.32) is shown space-filling for orientation. Side chains of amino acids that are conserved in both guinea pig and human $H_1Rs$ are not shown in this view. Amino acids of which side chains are depicted differ between human and guinea pig $H_1Rs$. 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 $H_1Rs$ as recently demonstrated using a GTPase assay (Seifert et al., 2003).

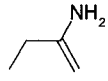
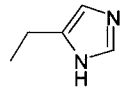
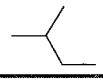
The species-dependent pharmacology of several of the histaprodifen analogs is also observed in functional assays. Measuring the effects on the contraction of the guinea pig ileum, HP-HA is up to 50-fold more potent than histamine (Christophe et al., 2003; Seifert et al., 2003), whereas in assays using heterologously expressed $hH_1Rs$, 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 $H_1R$: $Asp^{107}$ (3.32) in TM3 (Ohta et al., 1994; Moguilevsky et al., 1998; Nonaka et al., 1998; Bruysters et al., 2004), $Trp^{158}$ (4.56) in TM4 (Wieland et al., 1999), $Lys^{191}$ (5.39) and $Asn^{198}$ (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 $Phe^{432}$ (6.52) and $Phe^{435}$ (6.55) in TM6. None of these amino acids differ between human and guinea pig $H_1Rs$. 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 $Ile^{37}$ (1.42) and $Cys^{38}$ (1.43) in TM1, $Asn^{84}$ (2.61) and $Leu^{89}$ (2.66) in TM2, and $Leu^{449}$ (7.34) and $Ile^{459}$ (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 $H_1Rs$. To test this hypothesis, we created the following mutant $hH_1Rs$ in which the selected amino acids are individually replaced into

TABLE 1

Affinities of [$^3$H]mepyramine and expression levels of human and guinea pig $H_1Rs$ and several human $H_1R$ mutants

The values are determined by saturation [$^3$H]mepyramine binding assays. Data were calculated as the mean ± 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 | | | | |
|---|---|---|---|---|---|---|---|
| | $K_d$ | $B_{max}$ | Position | a.a. $hH_1R$ | | a.a. $gpH_1R$ | |
| | *nM* | *pmol/mg* | | | | | |
| $hH_1R$ | 1.2 ± 0.3 | 12.1 ± 0.9 | | | | | |
| $gpH_1R$ | 0.5 ± 0.1 | 5.3 ± 0.8[a] | | | | | |
| $hH_1R$-$Ile^{37}Val$ | 1.7 ± 0.5 | 11.4 ± 1.5 | 1.42 | Ile |  | Val |  |
| $hH_1R$-$Cys^{38}Ser$ | 1.4 ± 0.1 | 17.1 ± 2.1 | 1.43 | Cys |  | Ser |  |
| $hH_1R$-$Asn^{84}Ser$ | 1.1 ± 0.3 | 6.5 ± 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 ± 0.1 | 9.9 ± 2.8 | 6.53 | Ile |  | Val |  |
| $hH_1R$-$Leu^{449}Val$ | 1.4 ± 0.2 | 9.7 ± 2.7 | 7.34 | Leu |  | Val |  |
| $hH_1R$-$Ile^{459}Leu$ | 1.0 ± 0.2 | 10.3 ± 1.5 | 7.44 | Ile |  | Leu |  |

a.a., amino acid; N.D., not determined.
[a] $P < 0.01$.
[b] $P < 0.05$.

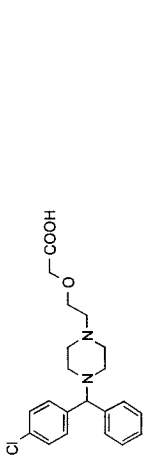
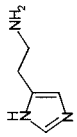
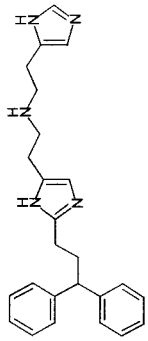
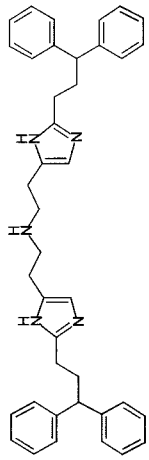
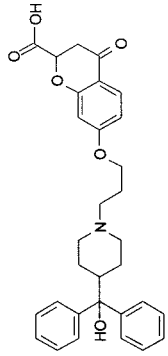
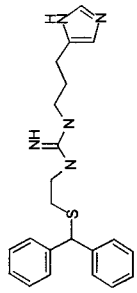
their guinea pig counterparts: $hH_1R$ $Ile^{37}Val$, $hH_1R$ $Cys^{38}Ser$, $hH_1R$ $Asn^{84}Ser$, $hH_1R$ $Leu^{89}His$, $hH_1R$ $Leu^{449}Val$, and $hH_1R$ $Ile^{459}Leu$. Although in our $H_1R$ model $Ile^{433}$ (6.53) points toward the plasma membrane, we also included the mutant $hH_1R$ $Ile^{433}Val$ receptor in our study because $Ile^{433}$ is located in between the established $hH_1R$-ligand interaction points $Phe^{432}$ (6.52) and $Phe^{435}$ (6.55). In general, we noticed that, at the selected positions, the amino acids present in the human $H_1R$ are bulkier than their guinea pig $H_1R$ counterparts (Table 1).

Most of the generated mutant $H_1Rs$ are expressed at comparable levels ($B_{max}$ values of ~10 pmol/mg) and bind [$^3$H]mepyramine with unchanged affinity ($K_d$ values of 0.5–1.7 nM) compared with wild-type human $H_1Rs$ (Table 1). However, the mutant receptor $hH_1R$-$Leu^{89}His$ (2.66), with a point mutation in the top of TM2, did not show any

TABLE 2

Binding affinities (p$K_i$) of $H_1R$ agonists for human and guinea pig $H_1Rs$ and several human $H_1R$ mutants

The p$K_i$ values are determined by [$^3$H]mepyramine displacements. Data were calculated as the mean ± S.E.M. of at least three experiments.

| | Cetirizine | Histamine | HP-HA | HP-HP | VUF 4669 | VUF 8401 |
|---|---|---|---|---|---|---|
| |  |  |  |  |  |  |
| $hH_1R$ | 7.9 ± 0.1 | 4.3 ± 0.2 | 6.1 ± 0.1 | 6.8 ± 0.2 | 7.7 ± 0.1 | 6.0 ± 0.1 |
| $gpH_1R$ | 7.7 ± 0.1[a] | 4.4 ± 0.2 | 7.1 ± 0.1[b] | 7.4 ± 0.1[a] | 9.0 ± 0.1[b] | 6.9 ± 0.1[b] |
| $hH_1R$ $Ile^{37}Val$ | 7.7 ± 0.1 | 3.9 ± 0.2 | 5.9 ± 0.1 | 6.9 ± 0.1 | 8.0 ± 0.1 | 5.9 ± 0.1 |
| $hH_1R$-$Cys^{38}Ser$ | 7.9 ± 0.1 | 3.9 ± 0.1 | 6.1 ± 0.1 | 6.8 ± 0.1 | 8.0 ± 0.1 | 6.0 ± 0.1 |
| $hH_1R$-$Asn^{84}Ser$ | 7.5 ± 0.1[b] | 4.2 ± 0.1 | 6.8 ± 0.1[b] | 7.3 ± 0.1[a] | 8.9 ± 0.1[b] | 6.4 ± 0.1[a] |
| $hH_1R$-$Ile^{433}Val$ | 8.0 ± 0.1 | 4.5 ± 0.2 | 6.2 ± 0.1 | 7.1 ± 0.1 | 8.0 ± 0.1 | 6.1 ± 0.1 |
| $hH_1R$-$Leu^{449}Val$ | 7.9 ± 0.1 | 4.0 ± 0.1 | 6.0 ± 0.1 | 7.0 ± 0.1 | 8.1 ± 0.1 | 6.0 ± 0.1 |
| $hH_1R$-$Ile^{459}Leu$ | 8.0 ± 0.1 | 4.4 ± 0.1 | 6.0 ± 0.1 | 7.0 ± 0.1 | 7.8 ± 0.2 | 6.0 ± 0.1 |

[a] $P < 0.05$.
[b] $P < 0.01$ versus $hH_1R$.

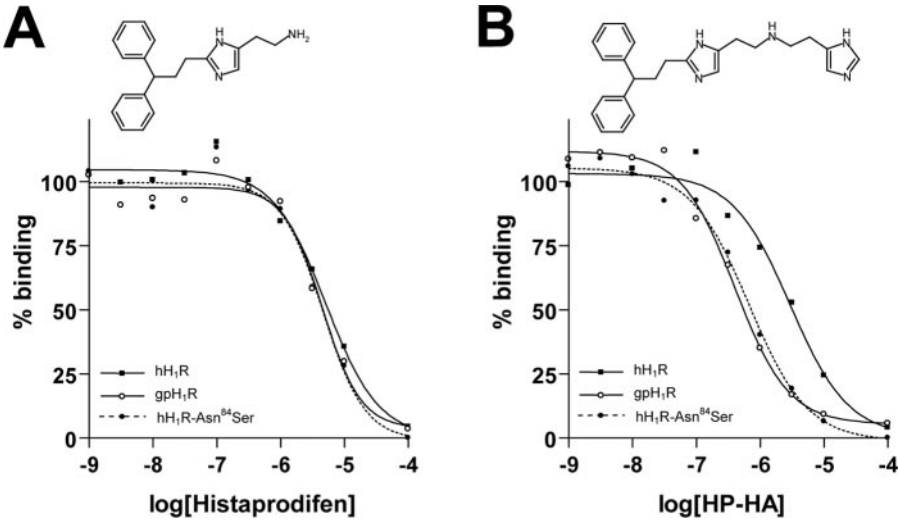



**Fig. 3.** Displacement of [$^3$H]mepyramine binding to wild-type $hH_1Rs$ (■) and $gpH_1Rs$ (○) and to the mutant receptor $hH_1R$ $Asn^{84}Ser$ (●) by histaprodifen (A) and the HP-HA dimer (B). A representative experiment is shown.

[$^3$H]mepyramine binding at concentrations up to 30 nM and may not be properly expressed at the cell membrane. Displacement of [$^3$H]mepyramine binding indicated that all tested mutant $H_1$Rs bind the endogenous agonist histamine with unchanged affinity (Table 2). Only for mutant $hH_1R$ $Asn^{84}Ser$ (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 $hH_1R$ ($pK_i$ = 6.1), resulting in a $gpH_1R$-like ($pK_i$ = 7.1) pharmacology (Fig. 3; Table 2). In addition, for HP-HP, the species difference was reversed by the $Asn^{84}Ser$ mutation (Table 2).

HP-HA is an agonist for the $hH_1R$ as measured using a $G\alpha_{q/11}$-mediated nuclear factor-κB reporter gene assay ($pEC_{50}$ = 6.4 ± 0.1) with a potency comparable with histamine ($pEC_{50}$ = 6.4 ± 0.2) (Bruysters et al., 2004). For both the $gpH_1R$ and mutant $hH_1R$ $Asn^{84}Ser$ (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 $H_1R$ pharmacology, and the mutant $hH_1R$ $Asn^{84}Ser$ not only displays a guinea pig $H_1R$ binding profile but also a guinea pig $H_1R$ functional profile.

These data suggest that residue $Asn/Ser^{84}$ (2.61) is of critical importance for the observed species-dependent agonist pharmacology of the human and guinea pig $H_1$Rs. Moreover, these data also indicate that for some $H_1R$ agonists TM2 is part of the $H_1R$ ligand binding-pocket. Interestingly, both human and rat $H_1$Rs 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 $H_1$Rs 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 $Asn/Ser^{84}$ (2.61) in the species-dependent $H_1R$ pharmacology. Consequently, pharmacological observations with rat $H_1$Rs will have more predictive power for the action of ligands at human $H_1$Rs.

**Characterization of a Novel, Species-Selective $H_1R$ Antagonist.** The $H_1R$ species-selective interactions were originally observed for bulky $H_1R$ agonists (HP-HA and HP-HP). These compounds seem to interact with the "classical" binding pocket (TMs 3, 4, 5, and 6) and $Asn/Ser^{84}$ (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 $H_1R$ antagonists. From this selection, VUF 4669 was identified as an $H_1R$ antagonist,

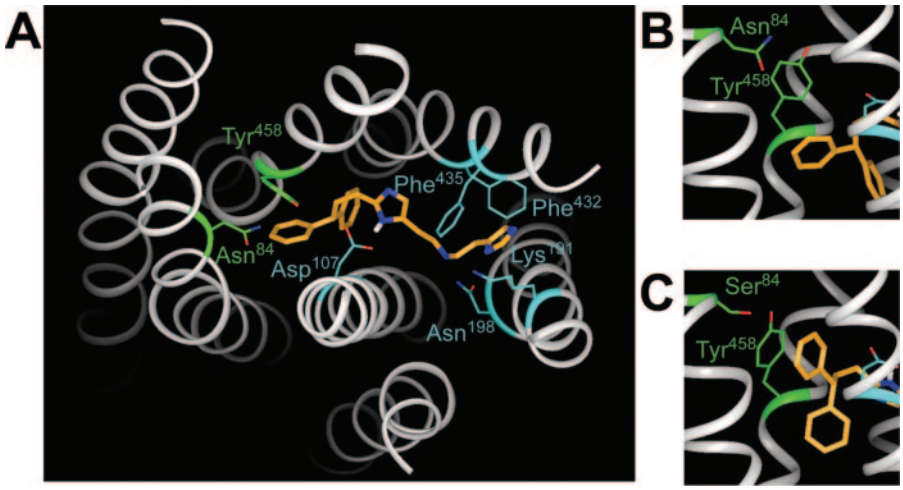



**Fig. 4.** Binding models of HP-HA (orange) in the human $H_1R$. View from the extracellular side (A) of the wild-type $hH_1R$. Detailed snapshot of view parallel to TM domains of wild-type (B) and mutant $hH_1R$ $Asn^{84}Ser$ (C). Amino acids previously known to interact with $H_1R$ ligands are depicted in blue [$Asp^{107}$ (3.32), $Lys^{191}$ (5.39), $Asn^{198}$ (5.46), $Phe^{432}$ (6.52) and $Phe^{435}$ (6.55)], and $Asn/Ser^{84}$ (2.61) and $Tyr^{458}$ (7.43) are depicted in green.

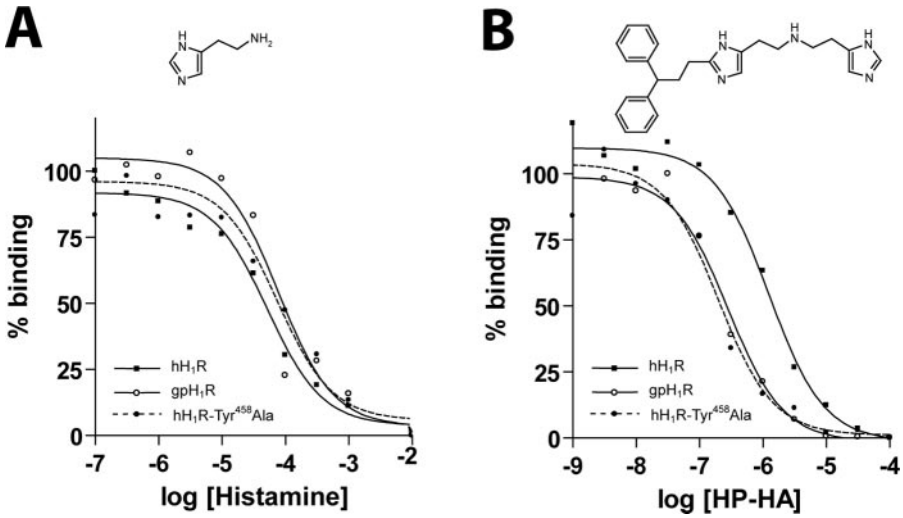



**Fig. 5.** Displacement of [$^3$H]mepyramine binding to wild-type $hH_1Rs$ (■) and $gpH_1Rs$ (○) and to the mutant receptor $hH_1R$ $Tyr^{458}Ala$ (●) by histamine (A) and HP-HA dimer (B). A representative experiment is shown.

which differentiates significantly between human and guinea pig $H_1$Rs. VUF 4669 showed a 17-fold increase in binding affinity for the guinea pig $H_1R$ ($pK_i = 9.0 \pm 0.1$), compared with its affinity for the human $H_1R$ ($pK_i = 7.7 \pm 0.1$) (Table 2). Apparently, the concept of species-selective binding is not restricted to $H_1R$ agonists but can also be observed for certain $H_1R$ antagonists. Again, VUF 4669 exhibits an increased affinity for the mutant $hH_1$ $Asn^{84}Ser$ receptor ($pK_i = 8.9 \pm 0.1$), confirming the guinea pig-like pharmacological profile of this mutant human $H_1R$. The other human to guinea pig $H_1R$ mutants used in this study exhibit an affinity for VUF 4669 that is identical to the affinity for the WT human $H_1R$ (Table 2).

Previously, also several arpromidine analogs, which display both $H_1R$ antagonistic and $H_2R$ agonistic properties, were characterized as guinea pig $H_1R$-preferring compounds (Seifert et al., 2003). Indeed, VUF 8401, a structural analog of arpromidine displays a 9-fold higher affinity for the guinea pig $H_1R$ than for the human $H_1R$ (Table 2). In addition, VUF 8401 binds with an increased affinity to the mutant $hH_1R$ $Asn^{84}Ser$ (2.61) (Table 2), although this mutation did not fully reverse the species difference. None of the other mutant $hH_1$Rs show an increased affinity for VUF 8401 (Table 2). Interaction with $Asn/Ser^{84}$ (2.61) therefore partially explains the observed species difference. We hypothesize that for arpromidine-like ligands the higher affinity depends on $Ser^{84}$ (2.61) and additional guinea pig $H_1R$-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. $H_1R$ antagonists such as terfenadine, fexofenadine, and oxatomide, 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 $Asn^{84}$ (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 $H_1R$ 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 $H_1R$ homology model suggests hydrogen bonding between $Asn^{84}$ (2.61) and $Tyr^{458}$ (7.43), a residue that is conserved between human and guinea pig $H_1$Rs. 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 $H_1R$ 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 $Asn^{84}$ (2.61) into Ser, thus creating a model of the $hH_1R$ $Asn^{84}Ser$ receptor containing HP-HA ($Asn^{84}Ser$ model). Molecular dynamics simulations were subsequently performed to optimize both HP-HA containing WT and $Asn^{84}Ser$ models. During both simulations, hydrogen bonding was maintained between $Asn^{84}$ (2.61) and $Tyr^{458}$ (7.43) in the WT model (3.31 Å; Fig. 4B) and between $Ser^{84}$ (2.61) and $Tyr^{458}$ (7.43) in the $Asn^{84}Ser$ model (2.80 Å; Fig. 4C). However, the orientation of $Tyr^{458}$ 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 $Asn^{84}Ser$ $H_1R$, the conformation of HP-HA in the $Asn^{84}Ser$ model is considered more favorable. In the WT model, $Tyr^{458}$ occupies the space that in the $Asn^{84}Ser$ model is occupied by one of the phenyl rings of HP-HA. Our computational studies therefore suggest that $Tyr^{458}$ might sterically hinder the binding of HP-HA in the $hH_1R$, thereby "forcing" HP-HA to bind deeper within the receptor.

To test the potential involvement of $Tyr^{458}$ (7.43) in the binding of HP-HA to the human $H_1R$, we mutated $Tyr^{458}$ (7.43) in the human $H_1R$ into an alanine ($hH_1R$ $Tyr^{458}Ala$). Saturation binding analysis using [$^3$H]mepyramine shows that this mutant $H_1R$ is expressed at comparable levels ($B_{max} = 8.2 \pm 3.5$ pmol/mg protein) and with an unchanged affinity for [$^3$H]mepyramine ($K_d = 3.0 \pm 0.7$) in comparison with the wild-type $H_1R$. The $Tyr^{458}Ala$ mutation did also not affect the affinity for histamine ($pK_i = 4.4 \pm 0.2$) (Fig. 5). Because the mutation $Tyr^{458}Ala$ would remove potential steric hindrance between HP-HA and the $hH_1R$, we expected an increased affinity of HP-HA. Indeed, mutation of $Tyr^{458}$ 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 $H_1R$ (Fig. 5). This affinity is similar to the affinity of HP-HA for both the $gpH_1R$ ($pK_i = 7.1 \pm 0.1$) and the $hH_1R$ $Asn^{84}Ser$ ($pK_i = 6.8 \pm 0.1$) (Table 2).

The results of our computational and mutagenesis studies indicate an important role of $Asn^{84}$ (2.61) as selectivity switch. Moreover, our results illustrate the first structural features of an additional binding pocket between TM2 and TM7 in the $H_1R$. 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 $H_2R$ agonists were suggested to interact with TM7 in the $H_2$ receptor (Kelley et al., 2001), whereas dopamine $D_2/D_4$ 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 $\beta_1/\beta_2$ 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 $hH_1R$ $Tyr^{458}$) are demonstrated to be involved in ligand binding to $5HT_{2A}$ (Roth et al., 1997) and muscarinic acetylcholine $M_3$ receptors (Wess et al., 1991). The involvement of TMs 2 and 7 in the $H_1R$ binding pocket of some $H_1R$ ligands is therefore highly likely.

## Conclusions

In conclusion, the human and guinea pig $H_1$Rs 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 $Asn^{84}$ (2.61) in the $hH_1R$ versus $Ser^{84}$ (2.61) in the $gpH_1R$. Based on molecular dynamics simulations and site-directed mutagenesis data, we suggest a possible role for $Tyr^{458}$ (7.43) in the binding of certain $H_1R$ ligands. Our data provide the first evidence that for these $H_1R$ ligands, TM2 and TM7 are also part of the ligand binding pocket. Exploitation of these additional interaction points within the $H_1R$ ligand binding pocket in drug development programs may

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

**Acknowledgments**

We thank F. Aelbrecht, C.v.d. Perren, G. J. Sterk, and J. Hulshof for valuable assistance.

**References**

Bakker RA, Schoonus SB, Smit MJ, Timmerman H, and Leurs R (2001) Histamine $H_1$-receptor activation of nuclear factor-$\kappa$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) 8*R*-lisuride is a potent stereospecific histamine $H_1$-receptor partial agonist. *Mol Pharmacol* **65:**538–549.

Ballesteros J and Weinstein H (1995) Intergrated 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 $H_1$-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 $H_1$-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 $H_1$-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 $H_1$ 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 $H_1$ histamine receptors: contribution of $Lys^{191}$ and $Thr^{194}$. *Mol Pharmacol* **61:**391–399.

Goldman LA, Cutrone EC, Kotenko 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 $\beta_1$- and $\beta_2$-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 $H_2$-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) $Lysine^{200}$ located in the fifth transmembrane domain of the histamine $H_1$ receptor interacts with histamine but not with all $H_1$ 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 $H_1$-receptor reveals a selective interaction of $asparagine^{207}$ with subclasses of $H_1$-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) $N^{\alpha}$-Imidazolylalkyl and pyridylalkyl derivatives of histaprodifen: synthesis and in vitro evaluation of highly potent histamine $H_1$-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 $H_1$ 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 $H_1$ 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 $H_1$ 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 $H_1$ receptor: roles of $aspartic\ acid^{107}$, $asparagine^{198}$ and $threonine^{194}$. *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-hydroxytryptamine2A 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 $H_1$-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 $H_1$-receptor antagonist binding site. A stereoselective pharmacophoric model based upon (semi-)rigid $H_1$-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 $H_1$ receptor. *J Biol Chem* **274:**29994–30000.

Zhang M-Q, Leurs R, and Timmerman H (1997) Histamine $H_1$-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 $H_1$-receptor agonists. *Prog Drug Res* **44:**49–85.

**Address correspondence to:** Prof. Dr. R. Leurs, Leiden/Amsterdam Center for Drug Research, Faculty of Sciences, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. E-mail: r.leurs@few.vu.nl