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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, The Netherlands (M.B., A.J., F. vd. M., R.A.B., and R.L.).

UCB S.A. Pharma Sector, Braine-l'Alleud, Belgium (M.G. and P.C.).

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b) Corresponding author:

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.

Tel: ++31 20 5987579

Fax: ++31 20 5987610

E-mail: r.leurs@few.vu.nl

c)

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d) List of non-standard abbreviations

TM	transmembrane domain
H_1R	histamine H_1 receptor
GPCRs	G-protein coupled receptors
COS-7	African green monkey kidney cells
TF	2-(3-trifluoromethylphenyl)histamine
MeHP	N^{α}-Methylhistaprodifen
HP-HA	Histaprodifen-histamine dimer
HP-HP	Histaprodifen-histaprodifen dimer

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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 (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 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 2 (TM2) appeared to act as a selectivity switch in the H_1R. Molecular modeling and site-directed mutagenesis studies suggests 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 limited to TMs 3, 4, 5 and 6, but also comprises an additional pocket formed by TMs 2 and 7.

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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, non-sedating 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 (TF) 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 ω,ω-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^{α} position to *e.g.* another histamine moiety (HP-HA) (Menghin et al., 2003). Compared to 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).

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Contrary to the potencies at either the guinea pig ileum and trachea or rat aorta (Christophe et al., 2003; Elz et al., 2000; Seifert et al., 2003), the potencies of various histaprodifen analogues (histaprodifen, MeHP, HP-HA and HP-HP) at human H_1Rs are at best similar to the potency of the endogenous ligand histamine (Bruysters et al., 2004; Seifert et al., 2003), indicating a potential species difference at the level of the H_1R recognition of these H_1R agonists.

In aminergic GPCRs, including the H_1R, the ligand-binding pocket is thought to reside in a hydrophilic cleft formed by the 7 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_1R Asp^{107} in TM3 (residue 3.32 according to the Ballesteros-Weinstein numbering) is part of the binding pocket of both H_1R agonists and antagonists (Bruysters et al., 2004; Moguilevsky et al., 1998; Nonaka et al., 1998; Ohta et al., 1994). Several additional amino acids in TM5 and TM6 are part of the H_1R binding pocket of histamine: Lys^{191} (5.39) (Bruysters et al., 2004; Gillard et al., 2002; Leurs et al., 1995; Moguilevsky et al., 1998; Wieland et al., 1999), Asn^{198} (5.46) (Bruysters et al., 2004; Leurs et al., 1994; Moguilevsky et al., 1995; Ohta et al., 1994) and Phe^{435} (6.55) (Bruysters et al., 2004) are considered to accommodate the imidazole ring of histamine. The H_1R 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) (Bruysters et al., 2004; Wieland et al., 1999).

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Recently, we studied the binding pocket of several histaprodifen analogues in the human H_1R (Bruysters et al., 2004). We demonstrated that histamine and the histamine moiety of histaprodifens bind to the human H_1R in a similar orientation. While the diphenylalkyl-system of histaprodifen interacts with the H_1R 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 appeared pivotal for agonist-induced activation of the hH_1R. 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_1Rs by a combined approach of molecular modeling and site-directed mutagenesis. We reevaluated several H_1R agonists and antagonists for their differences in affinity between human and guinea pig H_1Rs by [^{3}H]mepyramine displacement studies. Based on our knowledge of the H_1R binding site of the histaprodifens and the high (93%) level of sequence homology within the TM domains of the human and guinea pig H_1Rs, we extended our approach to mutant human H_1Rs in which selected amino acids were individually replaced by their guinea pig H_1R counterparts. Using this strategy, we identified Asn^{84} (2.61) in TM2 as the molecular basis for the observed species selectivity of certain H_1R ligands and discuss the implications of these findings for future drug design.

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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 ICN Biomedicals, Inc. (Zoetermeer, The Netherlands). Fexofenadine was purchased from Ultrafine Chemicals (Manchester,UK). VUF 4669 7-(3-(4-(hydroxydiphenylmethyl)piperidin-1-yl)propoxy)-4-oxochroman-2-carboxylic acid and VUF 8401 (3-(1H-imidazol-4-yl)propyl)-3-(2-(benzhydrylthio)ethyl)guanidine dipicrate were synthesized at the Vrije Universiteit Amsterdam, The Netherlands. Cetirizine dihydrochloride (Zyrtec®) and loratidine were synthesized at UCB SA, Braine l'Alleud, Belgium.

Gifts of 2-(3-trifluoromethylphenyl)HA dihydrogenmaleate, histaprodifen (2-[2-(3,3-diphenylpropyl]imidazol-4-yl)ethanamine dihydrogenmaleate), methylhistaprodifen (N^{α}-methyl-histaprodifen dihydrogenoxalate), histaprodifen-histaprodifen dimer trihydrogenoxalate and histaprodifen-histamine dimer (N^{α}-[2-(1H-imidazol-4yl)-ethyl]-histaprodifen) trihydrogenoxalate) (Dr. Dr. W. Schunack), the cDNA encoding the human H_1R (Dr. H. Fukui), and the expression vector $pcDEF_3$ (Goldman et al., 1996) (Dr. J. Langer) are greatly acknowledged.

Cell culture media, penicillin, and streptomycin bovine serum (FBS) were purchased from BioWhittaker (Verviers, Belgium). Cell culture plastics were

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obtained from Greiner Bio-one (Wemmel, Belgium). [^{3}H]mepyramine (~20 Ci/mmol) from Amersham Biosciences (Roosendaal, The Netherlands).

Numbering scheme of GPCRs

The indexing method introduced by Ballesteros and Weinstein (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 through 7) in which the residue is located, 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_1R.

Site-directed mutagenesis

The cDNA encoding the human H_1R (Fukui et al., 1994) was subcloned in the pAlter plasmid (Promega), and point mutations were created according to manufacturer's protocol (Altered Sites® II, Promega). cDNAs of mutant and wild-type receptors were sub-cloned 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 μg/ml streptomycin and 10% (V/V) FBS. COS-7 cells were transiently transfected using the DEAE-dextran

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method as previously described (Bakker et al., 2001), using 5 μg plasmid DNA per million cells. Two days post-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. Thus obtained crude cell homogenates were stored at -80°C until further use.

H_1R binding studies

The COS-7 cell homogenates (~ 5 μg) were incubated for 60 min at 30 ºC in 500 μl binding buffer (50 mM Na_2/K-phosphate buffer (pH = 7.4)) containing 3 nM [^{3}H]mepyramine. The non-specific 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 H_1R 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

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structure to avoid a clash between the top of TM3 and TM2 (Lopez-Rodriguez et al., 2002). Due to 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 minimizations 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 v2.1 (Jones et al., 1997) applying default parameters. The complex of the $Asn^{84}Ser$ mutant receptor with the ligand was obtained by changing the appropriate residue in the WT receptor-ligand model to its guinea pig homologue. 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. Gradually, restraints were 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 steps initialization, 5000 steps production, 5000 steps cooling), the C_α atoms of the receptor are fixed to their position as is the ligand. In the second round (15000 steps production, 5000 steps cooling) the ligand is released and free to move.

All minimizations and molecular dynamics simulations were performed using Discover (Accelrys Inc. San Diego).

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Analytical methods

Protein concentrations were determined according to Bradford (Bradford, 1976), using bovine serum albumin as a standard. Binding data were evaluated by a non-linear, least squares curve-fitting procedure using GraphPad Prism 4® (GraphPad Software, Inc., San Diego, CA). Obtained pK_i, pEC_{50} and K_d values are expressed as mean ± S.E.M. of at least three independent experiments. Statistical analyses were carried out by non-paired Student´s *t*-test. *P* values < 0.05 were considered to indicate a significant difference ([a]: $P < 0.05$, [b]: $P < 0.01$, [c]: $P < 0.001$). Despite significance, differences in pK_i values are only considered relevant when the difference is at least 0.3 logunits.

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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®), loratidine (Claritin®), oxatomide (Tinset®), fexofenadine (Allegra®), astemizole, terfenadine and mepyramine). As shown in Figure 1, none of the tested H_1R 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 H_1R over the human H_1R, corroborating recent findings by Seifert and coworkers (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 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, TF 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

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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 analogues 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 appear to be equipotent (Bruysters et al., 2004; Seifert et al., 2003).

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 (Bruysters et al., 2004; Moguilevsky et al., 1998; Nonaka et al., 1998; Ohta et al., 1994), Trp^{158} (4.56) in TM4 (Wieland et al., 1999), Lys^{191} (5.39) and Asn^{198} (5.46) in TM5 (Bruysters et al., 2004; Leurs et al., 1995; Leurs et al., 1994; Moguilevsky et al., 1998; Moguilevsky et al., 1995; Ohta et al., 1994), 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 (Figure 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

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either in close proximity to the hydrophilic cleft in the hH_1R, or on the interface of two TMs (Figure 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 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 towards the plasma membrane, we also included the mutant hH_1R Ile^{433}Val receptor in our study since 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) when compared to 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 [^{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_1Rs 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 to the wild-type hH_1R (pK_i = 6.1), resulting in a gpH_1R-

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like (pK_i = 7.1) pharmacology (Figure 3, Table 2). Also 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 NF-κB reporter gene assay (pEC_{50}= 6.4 ± 0.1) with a potency comparable to 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_1Rs. 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_1Rs have an asparagine at position 2.61. Measuring endothelium-dependent relaxation of rat aortic rings, Menghin and coworkers (Menghin et al., 2003) have shown that MeHP and HP-HA are equipotent, corroborating our previous findings with human H_1Rs 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_1Rs will have more predictive power for the action of ligands at human H_1Rs.

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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, HP-HP). These compounds appear 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, which differentiates significantly between human and guinea pig H_1Rs. VUF 4669 showed a 17-fold increase in binding affinity for the guinea pig H_1R (pK_i = 9.0 ± 0.1), compared to its affinity for the human H_1R (pK_i = 7.7 ± 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 ± 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 analogues, 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 analogue of arpromidine displays a 9-fold higher affinity for the guinea pig H_1R than for the human H_1R (Table 2). Also VUF 8401 binds with an increased affinity to the mutant hH_1R Asn^{84}Ser (2.61) (Table 2), although

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this mutation did not fully reverse the species difference. None of the other mutant hH_1Rs 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 analogues 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 like terfenadine, fexofenadine and oxatomide, however, also show such features, but appear not to be species-selective. Clearly, the simple presence of 2 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 (Okada et al., 2002; Palczewski et al., 2000). 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_1Rs. Using the automated docking procedure GOLD v2.1 (Jones et al., 1997) we subsequently docked the agonist HP-HA in the receptor model (Figure 4A). In contrast to H_1R antagonists such as cetirizine, the diphenyl moiety of HP-HA is not oriented towards TM6, but predicted to

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point towards 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, Figure 4B), and between Ser^{84} (2.61) and Tyr^{458} (7.43) in the $Asn^{84}Ser$ model (2.80 Å, Figure 4C). However, the orientation of Tyr^{458} differs between both models, probably due to the structural differences between Ser and Asn at position 2.61 (*e.g.* length and flexibility of the side chain). Since 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 ± 3.5 pmol/mg protein) and with an unchanged affinity for [^{3}H]mepyramine (K_d = 3.0 ± 0.7) in comparison to the wild-type H_1R. The $Tyr^{458}Ala$ mutation did also not affect the affinity for histamine (pK_i = 4.4 ± 0.2) (Figure 5). Since the mutation $Tyr^{458}Ala$ would remove potential steric hindrance between HP-HA

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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 ± 0.1) compared to the wild-type H_1R (Figure 5). This affinity is similar to the affinity of HP-HA for both the gpH_1R (pK_i = 7.1 ± 0.1) and the hH_1R $Asn^{84}Ser$ (pK_i = 6.8 ± 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 6 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 appears 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_1Rs exhibit significantly different affinities for agonists, like HP-HA and HP-HP, as well as for several

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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 to the currently known ligands.

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Legends for Figures

Figure 1. Binding affinities (pK_i) of a variety of histaminergic agonists (closed symbols) and antagonists (open symbols) for the human and guinea pig H_1Rs. The pK_i values for both wild-type human and guinea pig H_1Rs were determined by [^{3}H]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 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.

Figure 2. Snake plot (**A**) and top view (**B**) of the human H_1R showing the topology of the TM helices. Panel A: amino acids that are conserved in both guinea pig and human H_1Rs are depicted in white. Residues indicated in grey and black differ between human and guinea pig H_1Rs. The third intracellular loop is largely omitted from this graph (as indicated by 183 a.a.) since sequence homology in this region is very low. The residues in black have been selected (see text) in this study for a mutagenesis approach. Panel 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.

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Figure 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 histaprodifen-histamine dimer (HP-HA, **B**). A representative experiment is shown.

Figure 4. Binding models of HP-HA (orange) in the human H_1R. View from the extracellular side (Panel A) of the wild-type hH_1R. Detailed snapshot of view parallel to TM domains of wild-type (Panel B), and mutant hH_1R Asn^{84}Ser (Panel 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)), Asn/Ser^{84} (2.61) and Tyr^{458} (7.43) are depicted in green.

Figure 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 histaprodifen-histamine dimer (HP-HA, **B**). A representative experiment is shown.

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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. ND indicates that the value could not be determined. 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 (nM)	B_{max} (pmol/mg)	position	a.a. hH_1R	a.a. gpH_1R
hH_1R	1.2 ± 0.3	12.1 ± 0.9	-	-	-
gpH_1R	0.5 ± 0.1	5.3 ± 0.8[b]	-	-	-
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[a]	2.61	Asn	Ser
hH_1R-Leu^{89}His	> 30	ND	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]P<0.05, [b]P<0.01

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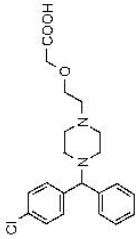
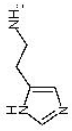
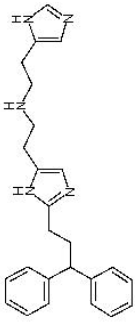
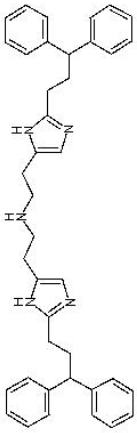
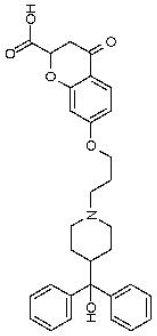
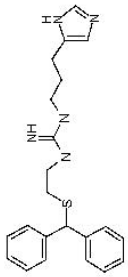
TABLE 2

Binding affinities (pK_i) of H_1R agonists for human and guinea pig H_1Rs and several human H_1R mutants. The pK_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[c]	7.4 ± 0.1[a]	9.0 ± 0.1[c]	6.9 ± 0.1[c]
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[c]	4.2 ± 0.1	6.8 ± 0.1[c]	7.3 ± 0.1[a]	8.9 ± 0.1[c]	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, [c]P<0.01 vs. hH_1R

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Figure 1

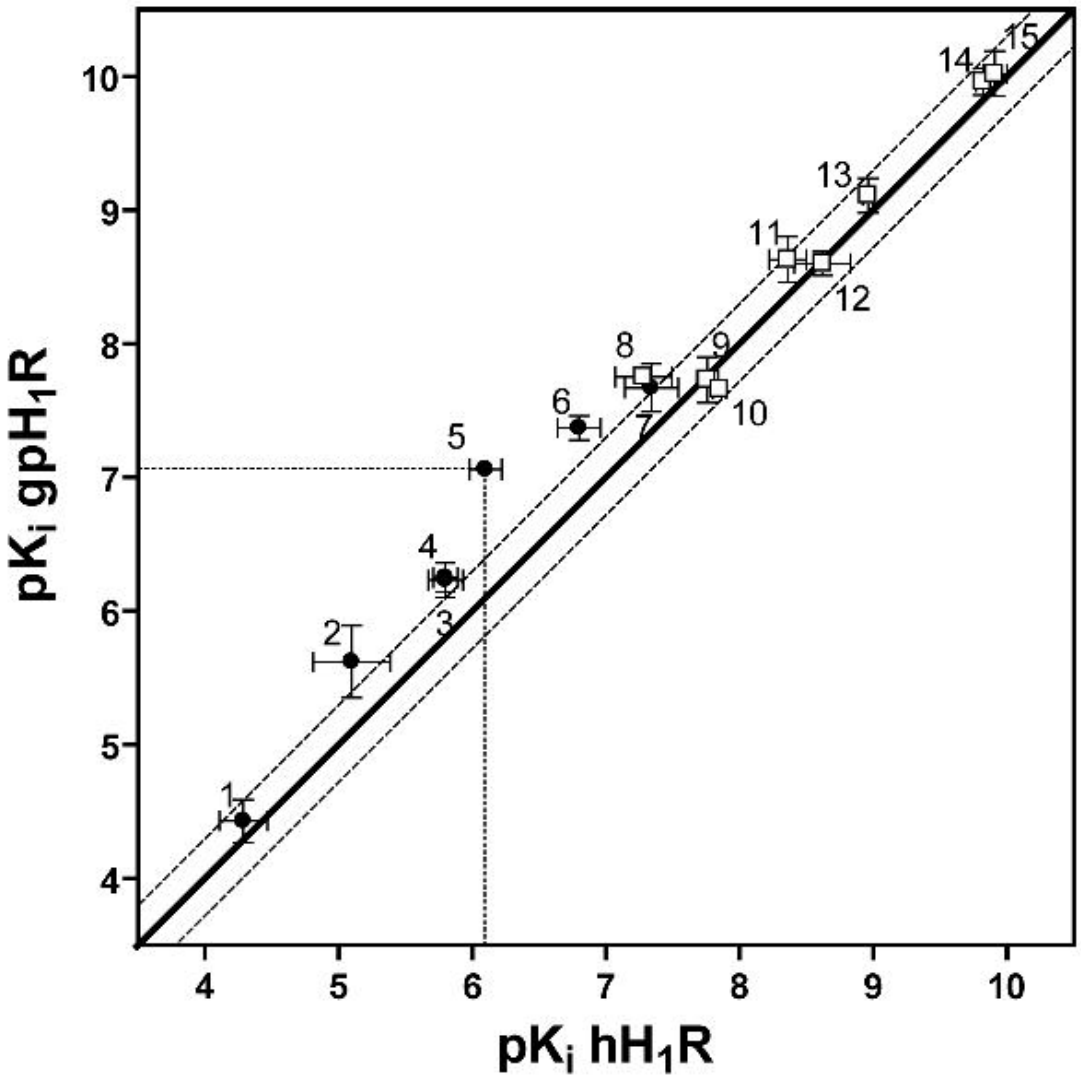

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Figure 2

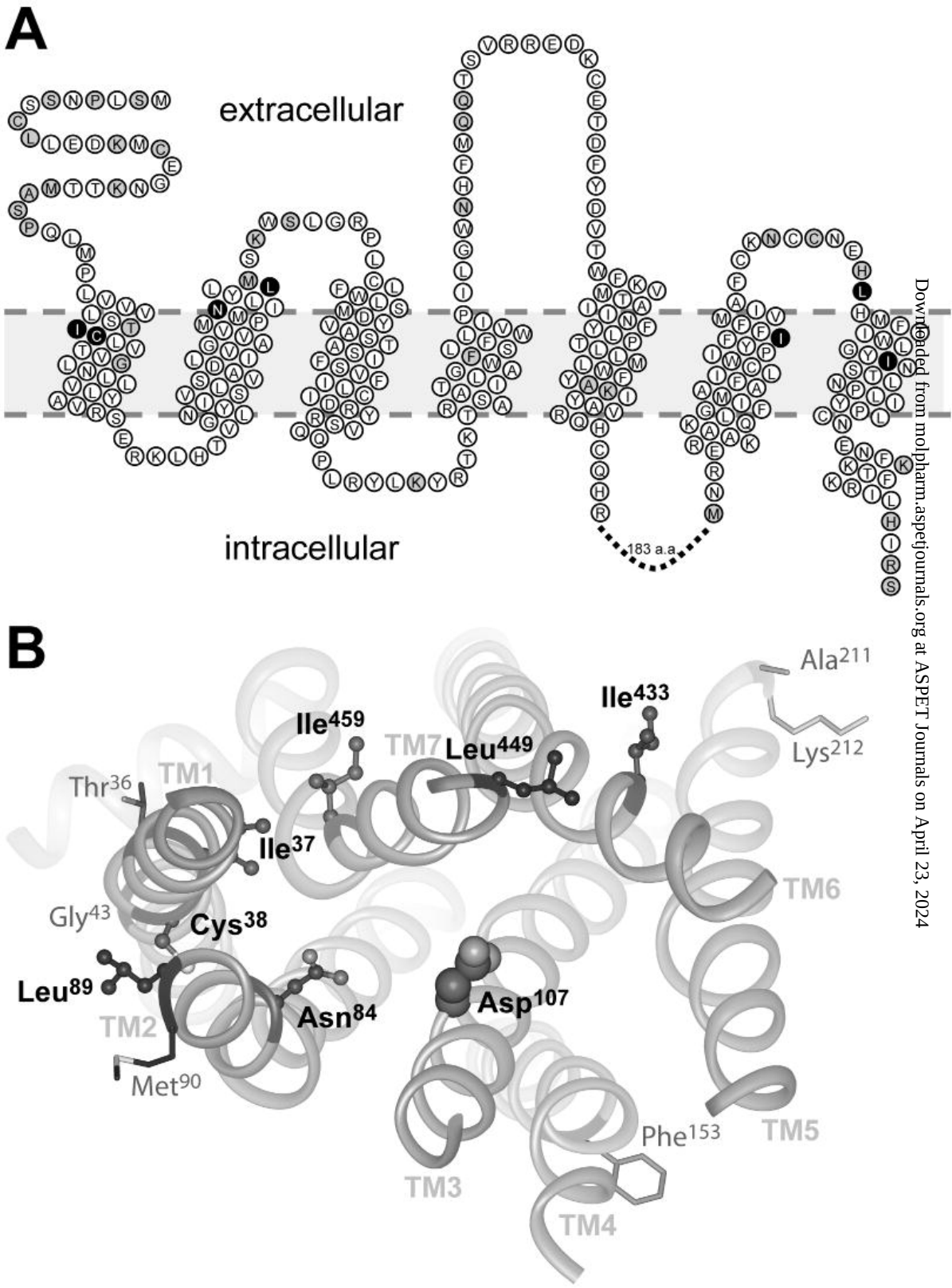

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Figure 3

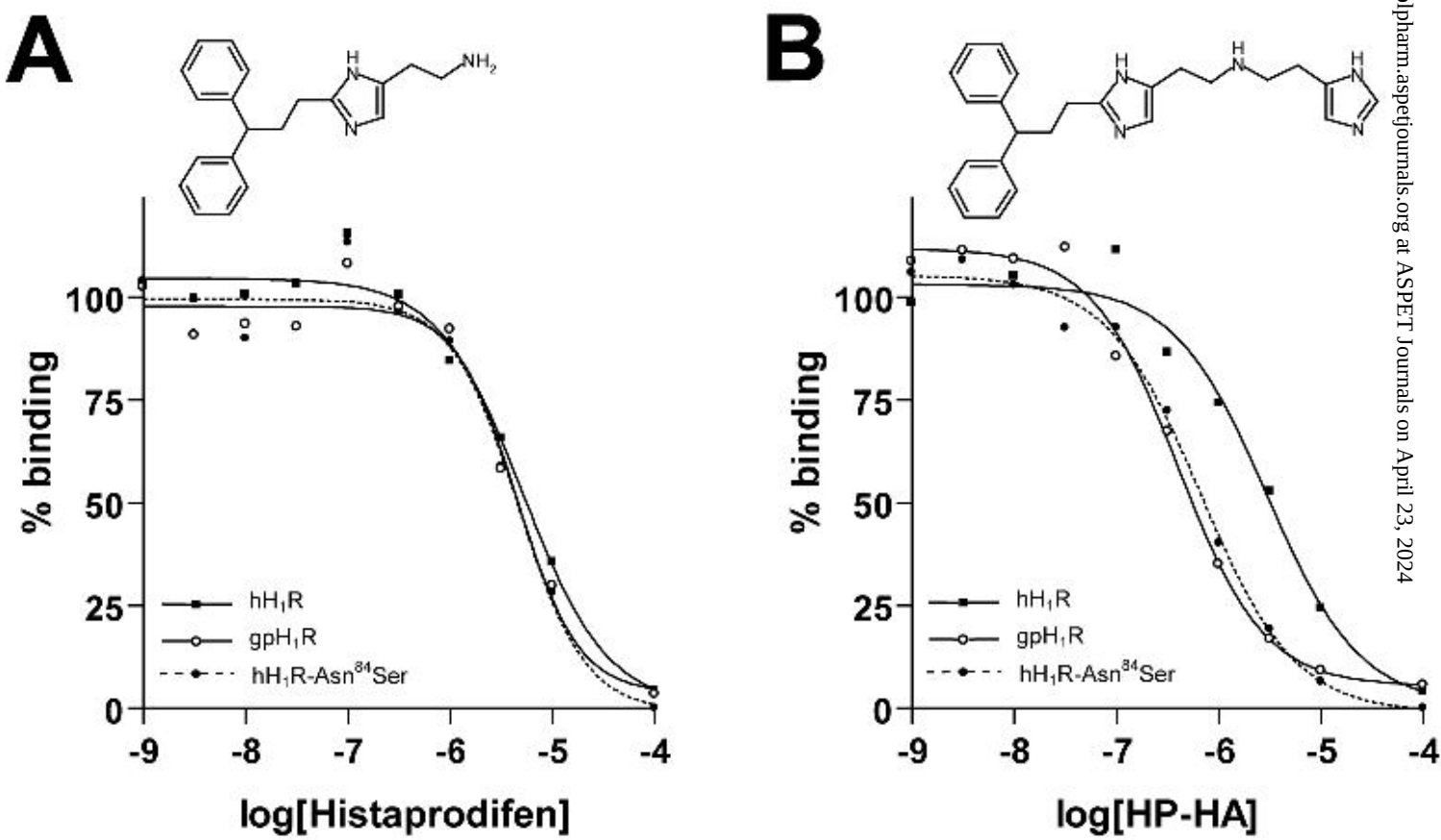

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Figure 4

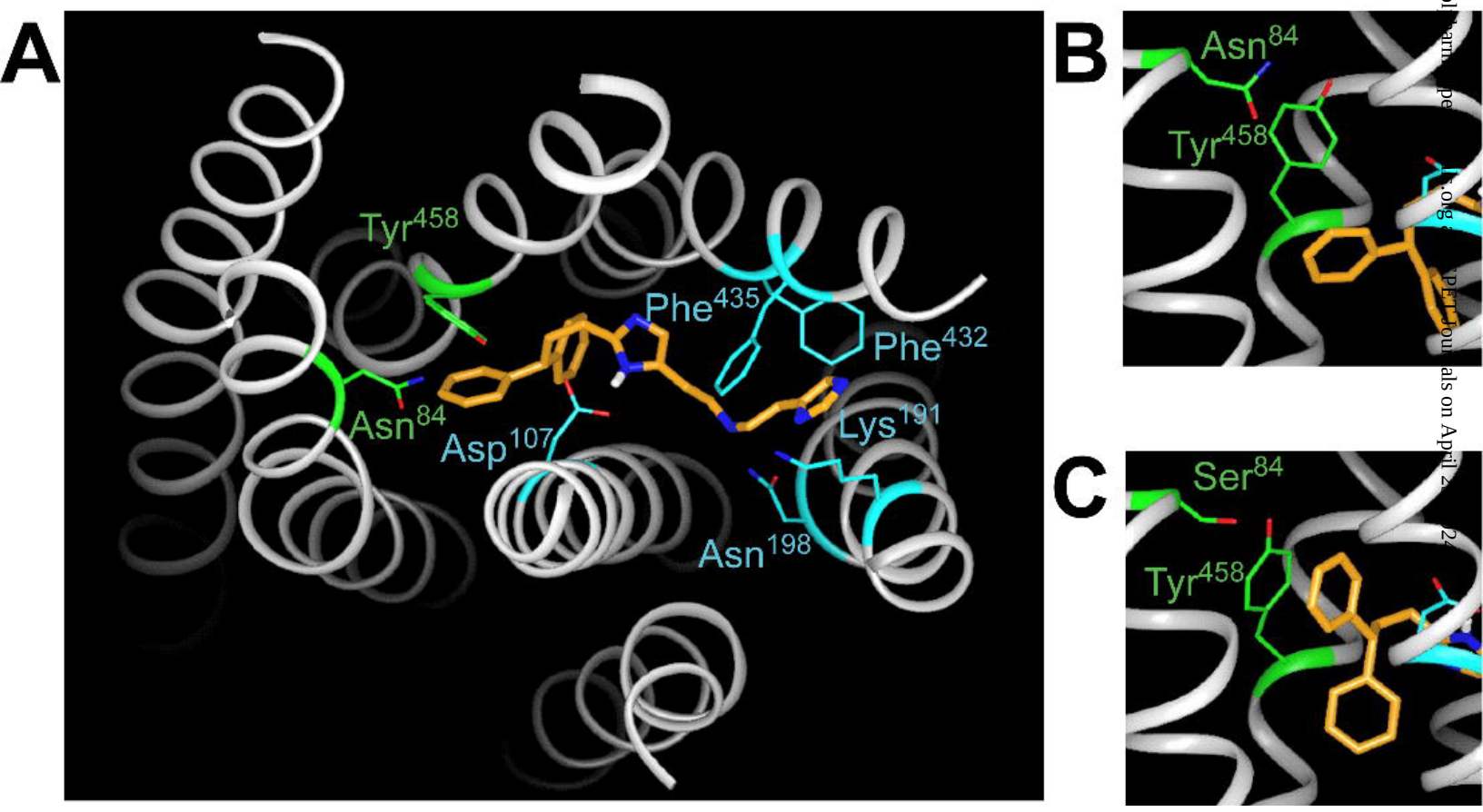

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Figure 5