

Multiple differences in agonist- and antagonist pharmacology between human and guinea pig histamine H₁-receptor

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Abbreviations used: GPCR, G-protein-coupled receptor; gpH₁R, guinea pig histamine H₁-receptor; gpH₂R, guinea pig histamine H₂-receptor; gpH₂R-G_{sαS}, fusion protein of the guinea pig histamine H₂-receptor and the short splice variant of G_{sα}; H_xR, histamine H₁-, H₂-, H₃- or H₄-receptor; hH₁R, human histamine H₁-receptor; hH₂R, human histamine H₂-receptor; hH₂R-G_{sαS}, fusion protein of the human histamine H₂-receptor and the short splice variant of G_{sα}; hH₁R-F153L, human histamine H₁-receptor bearing a Phe→Leu exchange at position 153; hH₁R-I433V, human histamine H₁-receptor bearing an Ile→Val exchange at position 433; hH₁R-F153L/I433V, human histamine H₁-receptor bearing a Phe→Leu exchange at position 153 and an Ile→Val exchange at position 433; RGS protein, regulator of G-protein signaling; TM transmembrane domain.

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Abstract

Species isoforms of histamine H₂-, H₃- and H₄-receptors differ in their pharmacological properties. The study aim was to dissect differences between the human H₁R (hH₁R) and guinea pig H₁R (ghH₁R). We co-expressed hH₁R and gpH₁R with regulators of G-protein signaling in Sf9 insect cells and analyzed the GTPase activity of G_q-proteins. Small H₁R agonists showed similar effects at hH₁R and gpH₁R, whereas bulkier 2-phenylhistamines and histaprodifens were up to ~10-fold more potent at gpH₁R than at hH₁R. Most 2-phenylhistamines and histaprodifens were more efficacious at gpH₁R than at hH₁R. Several first-generation H₁R antagonists were ~2-fold, and apromidine-type H₁R antagonists up to ~10-fold more potent at gpH₁R than at hH₁R. [³H]Mepyramine competition binding studies confirmed the potency differences of the GTPase studies. Phe-153→Leu-153- or Ile-433→Val-433 exchange in hH₁R (hH₁R→gpH₁R) resulted in poor receptor expression, low [³H]mepyramine-affinity and functional inactivity. The Phe-153→Leu-153/Ile-433→Val-433 double mutant expressed excellently but only partially changed the pharmacological properties of hH₁R. Small H₁R agonists and 2-phenylhistamines interacted differentially with human and guinea pig H₂R in terms of potency and efficacy, respectively. Our data show the following: *(i)* There are differences in agonist- and antagonist-pharmacology of hH₁R and gpH₁R encompassing diverse classes of bulky ligands. These differences may be explained by higher conformational flexibility of gpH₁R relative to hH₁R. *(ii)* Phe-153 and Ile-433 are critical for proper folding and expression of hH₁R. *(iii)* H₂R species isoforms distinguish between H₁R agonists.

Histamine serves as a neurotransmitter and autacoid and acts through specific H_xRs designated as H₁R, H₂R, H₃R and H₄R, respectively (Hill et al., 1997; Hough, 2001). The H₁R couples to G_q-proteins. Numerous H₁R agonists and antagonists are known. H₁R agonists are divided into three classes (Fig. 1); *i.e.* (i) small agonists (**2-4**) derived from histamine (**1**), (ii) histamine derivatives with bulkier aromatic substituents at the 2-position of the imidazole ring (**5-18**), and (iii) histaprodifens, *e.g.* compounds **19-23** (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000). H₁R agonists are important experimental tools to analyze H₁R function in cellular and organ systems (Zingel et al., 1995; Hill et al., 1997). H₁R antagonists are commonly divided into sedating (first-generation, **24-32**) and non-sedating (second-generation, **41-45**) antagonists (Fig. 2). Today, especially the second-generation H₁R antagonists are of great importance for the treatment of allergic diseases (Hill et al., 1997). Guanidines **33, 34** and **36-39** derived from arpromidine (**35**) are dual H₂R agonists/H₁R antagonists (Buschauer, 1989).

The availability of H_xR cDNAs allowed for the comparison of the pharmacological properties of H_xR species isoforms in recombinant systems under identical experimental conditions. Such expression studies uncovered species-differences in the pharmacological properties of hH₂R and gpH₂R (Kelley et al., 2001), rat and human H₃R (Ligneau et al., 2000; Lovenberg et al., 2000) and H₄R from mouse, rat, guinea pig and humans (Liu et al., 2001). Species-differences in the pharmacological properties of H_xRs provided opportunities to analyze the molecular basis of ligand/GPCR interactions (Ligneau et al., 2000; Kelley et al., 2001). From the standpoint of drug design, the pharmacological properties of hH_xRs are important because in the H_xR field, essentially all structures generated so far were derived from animal models, mostly from rat and guinea pig (Zingel et al., 1995; Hill et al., 1997).

The species-differences in pharmacological properties of H₂R, H₃R and H₄R raise the question whether this is a general characteristic of H_xR_s. In fact, the *K_d* values of [³H]mepyramine for H₁R_s from various species differ by ~2-6-fold (Chang et al., 1979). Moreover, histaprodifens exhibit different potencies and efficacies in the guinea pig ileum and rat aorta (Elz et al., 2000). Furthermore, 2-(3-chlorophenyl)histamine (**12**) is a potent H₁R agonist in the guinea pig ileum but failed to exhibit agonistic activity in H₁R-expressing dibutyryl cAMP-differentiated human HL-60 leukemia cells (Seifert et al., 1994). A snake plot of hH₁R depicts the relative positions and topology of amino acid residues in the TM domains, putative agonist- and antagonist binding sites, and differences with respect to the gpH₁R (Fig. 3). Mutagenesis data (Leurs et al., 1994, 1995; Ohta et al., 1994; Nonaka et al., 1998) and modeling approaches (Elz et al., 2000) indicated that histamine and histaprodifens interact with amino acid residues in TMs III, IV, V and VII. Considering the alignment of H₁R_s with bovine rhodopsin (Palczewski et al., 2000) and results of the substituted-cysteine accessibility method with the dopamine D₂-receptor (Ballesteros et al., 2001), there are no amino acid differences in the ligand binding pocket of gpH₁R and hH₁R. The two lipid-directed residues, Phe-153 in TM IV of hH₁R *versus* Leu in gpH₁R and Ile-433 in TM VI of hH₁R *versus* Val in gpH₁R, represent the only differences near the binding site. Although these amino acid exchanges are conservative, the amino acids in hH₁R are bulkier than those in gpH₁R, and such differences could have an impact on the ligand-binding pocket.

The aim of the present study was to compare recombinant hH₁R and gpH₁R expressed in Sf9 insect cells under identical experimental conditions. We also examined the roles of Phe-153 and Ile-433 in hH₁R function. As read-out, we focused on the determination of the GTPase activity of insect cell G_q-proteins in the presence of the RGS proteins RGS4 and GAIP. This co-

expression system provides a sensitive model for studying H₁R at the G-protein level (Houston et al., 2002). The GTPase assay is a steady-state method and eliminates the impact of effector availability/compartimentation and pharmacokinetic barriers on the properties of agonists (Buschauer, 1989; Ostrom et al., 2000). Moreover, we conducted [³H]mepyramine binding studies and analyzed the effects of H₁R agonists on recombinant H₂R-G_{sα} fusion proteins, recently verified as sensitive systems for the analysis of H₂Rs (Kelley et al., 2001).

Methods

Materials. Construction of the cDNAs for hH₁R-F153L, hH₁R-I433V and hH₁R-F153L/I433V was performed by overlap-extension PCR following recently described procedures (Houston et al., 2002). Dimethindene enantiomers were a kind gift of Dr. G. Lambrecht (Department of Pharmacology, University of Frankfurt/M., Germany). Ketotifen was a gift from Novartis (Basel, Switzerland), azelastine a gift from Asta Medica (Frankfurt/M., Germany), fexofenadine a gift from Janssen-Cilag (Neuss, Germany), and terfenadine a gift from Aventis (Frankfurt/M., Germany). Guanidines **33-38** were synthesized as described (Buschauer, 1989). Guanidine **39** was prepared by analogy to the procedures described for guanidines **33-38**. 2-Methylhistamine (**2**) and 2-(2-thiazolyl)ethanamine (**3**) were synthesized using standard procedures. Compounds **5-18** were prepared according to published procedures (Zingel et al., 1990; Leschke et al., 1995). Compounds **22, 23** and **40** were available by synthetic pathways reported for the synthesis of **19-21** (Elz et al., 2000). Structures of synthesized compounds were confirmed by elemental analysis (C, H, N), ¹H NMR spectroscopy and mass spectrometry. Purity of compounds was >98% as determined by high-performance liquid chromatography or capillary electrophoresis. Tunicamycin, histamine, betahistine, promazine, chlorpromazine, mianserin, cyproheptadine, diphenhydramine, mepyramine, triprolidine, and (+)-chlorpheniramine were from Sigma (St. Louis, MO). Sources of other materials are described elsewhere (Kelley et al., 2001; Houston et al., 2002).

Cell culture and membrane preparation. Recombinant baculoviruses encoding hH₁R-F153L, hH₁R-I433V and hH₁R-F153L/I433V were generated in Sf9 cells using the BaculoGOLD transfection kit (PharMingen, San Diego, CA) according to the manufacturer's instructions. Infection and culture of Sf9 cells and membrane preparation were performed as

described (Kelley et al., 2001; Houston et al., 2002). In some cultures, we added tunicamycin (10 $\mu\text{g/ml}$) to cultures to inhibit *N*-glycosylation of H₁Rs (Seifert and Wenzel-Seifert, 2001).

[³H]Mepyramine binding assay. Membranes expressing various H₁R constructs plus RGS proteins were thawed and sedimented by a 15 min centrifugation at 4°C and 15,000 x *g*. Membranes were resuspended in binding buffer (12.5 mM MgCl₂, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Tubes (total volume 500 μl) contained 20-25 μg of membrane protein. Incubations were conducted for 90 min at 25°C and shaking at 250 rpm. For H₁R saturation binding experiments, tubes contained 0.2-20 nM [³H]mepyramine (hH₁R, gpH₁R and hH₁R-F153L/I433V) or 2-100 nM [³H]mepyramine (hH₁R-F153L and hH₁R-I433V). Non-specific binding was routinely determined in the presence of 10 μM mepyramine (**30**). Non-specific binding in the presence of saturating concentrations of compounds **1**, **3**, **12**, **14**, **15**, **19**, **20**, **31**, **35** and **36** was virtually identical to non-specific binding in the presence of compound **30** (data not shown). Competition binding experiments were carried out in the presence of 2 nM [³H]mepyramine and unlabeled ligands at various concentrations. Bound [³H]mepyramine was separated from free [³H]mepyramine by filtration through GF/C filters, followed by three washes with 2 ml of binding buffer (4°C). Filter-bound radioactivity was determined by liquid scintillation counting.

Steady-state GTPase activity assay. Membranes expressing various H₁R constructs plus RGS proteins or H₂R-G_{s α} fusion proteins were thawed, sedimented and resuspended in 10 mM Tris/HCl, pH 7.4. Assay tubes contained Sf9 membranes (10 μg of protein/tube), 1.0 mM MgCl₂, 0.1 mM EDTA, 0.1 mM ATP, 100 nM GTP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg creatine kinase and 0.2% (w/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4, and H_xR ligands at various concentrations. Reaction mixtures (80 μl) were incubated for 3

min at 25°C before the addition of 20 µl [γ -³²P]GTP (0.2-0.5 µCi/tube). Reactions were conducted for 20 min at 25°C. Reactions were terminated by the addition of 900 µl slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH₂PO₄, pH 2.0. Charcoal-quenched reaction mixtures were centrifuged for 15 min at room temperature at 15,000 x g. Seven hundred µl of the supernatant fluid of reaction mixtures were removed, and ³²P_i was determined by liquid scintillation counting.

SDS-PAGE and immunoblot analysis. Membrane proteins were separated on SDS polyacrylamide gels containing 10% (w/v) acrylamide. Proteins were then transferred onto Immobilon-P transfer membranes (Millipore; Bedford, MA). Membranes were reacted with M1 antibody (1 : 1,000). Immunoreactive bands were visualized by sheep anti-mouse IgG (1 : 1,000) coupled to peroxidase, using *o*-dianisidine and H₂O₂ as substrates. Expression of RGS proteins was verified by immunoblot analysis with specific anti-RGS4 IgG and anti-GAIP IgG as described (Houston et al., 2002).

Miscellaneous. Protein concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). All analyses of experimental data were performed with the Prism 3.02 software (GraphPad-Prism, San Diego, CA). K_i - and K_B values were calculated according to Cheng and Prusoff (1973). Statistical comparisons were performed with the *t*-test.

Results

Immunological detection of H₁R constructs. The H₁R constructs analyzed in this study were all *N*-terminally tagged with the FLAG epitope, allowing immunological detection with the M1 monoclonal antibody (Houston et al., 2002). The predicted molecular mass of non-glycosylated hH₁R and gpH₁R is ~56 kDa (Fukui et al., 1994; Traiffort et al., 1994). The FLAG epitope-tagged hH₁R expressed in Sf9 membranes migrated as diffuse ~75 kDa doublet in SDS-PAGE (Figs. 4A and 4B). Treatment of Sf9 cells with the inhibitor of *N*-glycosylation, tunicamycin (Seifert and Wenzel-Seifert, 2001), shifted the majority of the protein towards 70 kDa and rendered the lower band crisper. Migration of FLAG epitope-tagged gpH₁R in SDS-PAGE differed considerably from the migration of hH₁R. In membranes expressing gpH₁R, faint and diffuse bands in the ~36 kDa- and ~50 kDa regions were detected, and tunicamycin treatment had little effect on migration of gpH₁R in SDS-PAGE (Fig. 4A). Additionally, we detected intense and crisp bands of ~16 kDa and ~30 kDa. Both hH₁R-F153L and hH₁R-I433V showed a broad ladder of diffuse bands ranging from ~30-80 kDa, and there was a more intense doublet at 28-29 kDa (Fig. 4B). The hH₁R-F153L/I433V double mutant showed the predicted migration in SDS-PAGE, *i.e.* this mutant migrated as a ~56 kDa band.

Analysis of H₁R constructs in [³H]mepyramine binding assays. The K_d of [³H]mepyramine for hH₁R expressed in Sf9 membranes was 1.8-fold higher than the K_d for gpH₁R (Table 1). The B_{max} values of hH₁R and gpH₁R expression in Sf9 membranes were similar to the expression levels reported for the β_2 -adrenoceptor (Seifert et al., 1998). Compared to hH₁R, the [³H]mepyramine-affinities of hH₁R-F153L and hH₁R-I433V were reduced by ~8-12-

fold, and the B_{max} values were reduced by ~5-6-fold. The double mutation restored [³H]mepyramine-affinity of hH₁R and efficient expression.

Potencies and efficacies of H₁R and H₂R agonists at H₁R constructs in the GTPase assay. We studied three classes of H₁R agonists in the GTPase assay (Fig. 1). As a control we also studied the H₂R agonists amthamine (**46**) and dimaprit (**47**) (Hill et al., 1997). Table 2 and Fig. 5 summarize the data for hH₁R and gpH₁R co-expressed with RGS4 and GAIP since no significant differences were observed between the two RGS proteins (data not shown). Only histamine and the small histamine derivatives **2** and **3** were full hH₁R agonists, whereas all other modifications resulted in reductions of efficacy. Additionally, compounds **2** and **3** were less potent hH₁R agonists than histamine. We identified only two agonists that were more potent at hH₁R than histamine, *i.e.* the histaprodifens **19** and **20**. However, the moderate increase in potency (1.8-2.7-fold) was accompanied by a significant decrease in efficacy. The introduction of a phenyl group (**6**) or particularly a benzyl group (**5**) at the 2-position of the imidazole ring substantially reduced agonist potency. Introduction of a halogen in the *meta* position of the phenyl ring partially restored agonist potency in the order F < Cl < Br ~ I (compare **6**, **9**, **12**, **14** and **15**). Other hydrogen-donating *meta* substituents (OMe, CF₃) were also favorable (**16** and **17**), whereas a methyl group (**7**) and halogen substitutions in the *ortho*- or *para* position of the phenyl ring (**8** and **13**) further reduced agonist potency. At hH₁R, histaprodifens **21-23** were less potent than histamine. The H₂R agonists **46** and **47** were essentially devoid of agonistic activity at the hH₁R (Table 2).

We did not observe significant differences in potency and efficacy of the small H₁R agonists **1-4** between hH₁R and gpH₁R (Table 2). This similarity between the H₁R isoforms is

reflected by a linear correlation of the pEC_{50} values of the small agonists at hH_1R and gpH_1R that is close to the theoretical correlation describing identity of H_1R species isoforms (Fig. 5A). However, when the effects of 2-phenylhistamines and histaprodifens were analyzed, significant differences between hH_1R and gpH_1R emerged. All compounds of these two classes were significantly more potent (3.2-9.9-fold) at gpH_1R than at hH_1R . The different interaction of 2-phenylhistamines and histaprodifens with hH_1R and gpH_1R is reflected by a linear correlation of the potencies of each series that is shifted towards the left relative to the theoretical correlation describing pharmacological identity of the GPCR species isoforms (Figs. 5B and 5C). These linear correlations also show that the overall structure/activity relationships of those compounds are similar at both H_1R species isoforms. In addition to the higher potency, most 2-phenylhistamines (**6**, **8-12**, **14-17**) and 3 out of 5 histaprodifens (**20**, **21**, **23**) were significantly more efficacious at gpH_1R than at hH_1R . Finally, the small H_2R agonist dimaprit (**47**) showed only minimal agonistic effects at gpH_1R , but another small agonist, amthamine (**46**), was a weak partial gpH_1R agonist with significantly higher efficacy at gpH_1R than at hH_1R .

We failed to detect GTPase stimulation by histamine and compounds **3** and **12** in Sf9 membranes expressing hH_1R -F153L and hH_1R -I433V plus RGS proteins (data not shown). In contrast, histamine and compound **3** stimulated GTP hydrolysis in membranes expressing hH_1R -F153L/I433V as potently and efficiently as in membranes expressing hH_1R or gpH_1R . 2-Substituted histamines and histaprodifens tended to be more potent and efficacious at hH_1R -F153L/I433V than at hH_1R , but only the potency and efficacy of compound **12** were significantly increased.

Constitutive activity of H₁Rs. hH₁R is constitutively active, and many first- and second-generation H₁R antagonists possess inverse agonistic activity (Bakker et al., 2001; Weiner et al., 2001). However, the extent of constitutive activity of hH₁R is dependent on the specific expression system. All first-generation H₁R antagonists (**24-32**), second-generation H₁R antagonists (**41-45**) and guanidines (**33-39**) examined exhibited only small inverse agonistic activity at hH₁R expressed in Sf9 membranes, *i.e.* the inhibitory effects of compounds amounted to ~5-15% of the stimulatory effect of histamine (data not shown). There were no significant differences in the inverse agonist effects of H₁R antagonists at hH₁R and gpH₁R. These data indicate that the constitutive activity of the two GPCR isoforms is similar.

Potencies of H₁R antagonists at H₁R constructs in the GTPase assay. In agreement with the [³H]mepyramine binding studies (Table 1), mepyramine (**30**) was about two-fold less potent at inhibiting histamine-stimulated GTP hydrolysis in membranes expressing hH₁R than in membranes expressing gpH₁R (Table 3). A similar difference in potency was observed for two other first-generation H₁R antagonists, triprolidine (**31**) and (+)-chlorpheniramine (**32**), whereas the other first-generation antagonists studied (**24-28**, dimethindene enantiomers (*R*)-(-)-**29** and (*S*)-(+)-**29**) did not exhibit significantly different potencies at hH₁R and gpH₁R. (*R*)-(-)-Dimethindene was ~30-40-fold more potent than (*S*)-(+)-dimethindene. The stereoselectivity of recombinant H₁Rs for dimethindene enantiomers is in accordance with data for the H₁R expressed in the guinea pig ileum (Pfaff et al., 1995). Among the second-generation H₁R antagonists **41-45**, no significant differences in potency between hH₁R and gpH₁R emerged.

Arpromidine (**35**) and arpromidine-derived guanidines (**33**, **34**, **36-38**) are not only very potent H₂R agonists but also moderately potent H₁R antagonists (Buschauer, 1989). The

H₁R-antagonistic properties of guanidines are explained by the structural similarity of compounds **33-38** and **30-32** (Fig. 2). Guanidines **33-38** inhibited histamine-stimulated GTP hydrolysis in Sf9 membranes expressing gpH₁R with K_B values of ~50-150 nM (Table 3). Guanidines **33-38** were all significantly more potent antagonists at gpH₁R than at hH₁R and showed greater gpH₁R/hH₁R selectivity than compounds **30-32**. The difference in potency was most pronounced (~9-fold) for compound **36** that is distinguished from the other guanidines by a *para*-Cl in the phenyl moiety (Fig. 2). In contrast, guanidine **39** that possesses a tri-chlorinated phenyl ring and a thiazole instead of a pyridyl ring (Fig. 2), did not discriminate between hH₁R and gpH₁R. Modifications of the substituents in guanidines **33-39** had a considerably larger impact on antagonist potency at gpH₁R (~7-fold) than at hH₁R (~2-fold).

In the 2-phenylhistamine derivative **40**, the free amino group of histamine was integrated into a piperidine ring (Fig. 2). This modification is predicted to interfere with the binding of the basic nitrogen to Asp-107 (hH₁R) (Ohta et al., 1994). In fact, compound **40** exhibited 6.5-8-fold reduced apparent affinity compared to its parent compound (**17**) (Fig. 1) at hH₁R and gpH₁R (Tables 2 and 3). Moreover, introduction of the piperidine ring into **17** conferred antagonistic properties to compound **40** (Table 3). This was also confirmed in the guinea pig ileum assay (K_B of compound **40**, 400 nM). Compound **40** was a several-fold more potent antagonist at gpH₁R than at hH₁R.

In agreement with the binding data (Table 1), mepyramine (**30**) was similarly potent at inhibiting histamine-stimulated GTP hydrolysis in Sf9 membranes expressing hH₁R and hH₁R-F153L/I433V (Table 3). The double mutation exhibited inconsistent effects on the potencies of guanidines **33** and **35-38** as well as of the 2-phenylhistamine derivative **40**. Specifically, the

F153L/I433V mutation increased the potency of **36** 1.5-fold, had no effect on the potency of **35** and **37** and decreased the potency of compounds **33**, **38** and **40** by up to 2-fold.

Affinities of H₁R agonists and antagonists at H₁R constructs in the [³H]mepyramine binding assay. Histamine and 2-(3-chlorophenyl)histamine (**12**) inhibited [³H]mepyramine binding in Sf9 membranes expressing hH₁R or gpH₁R plus RGS proteins according to a monophasic function that was not shifted to the right by guanosine 5'-O-(3-thiotriphosphate) (10 μM) (data not shown). Thus, we could not detect high-affinity agonist binding. These data were expected since there is a paucity of endogenous G-proteins relative to the expressed mammalian GPCRs in Sf9 membranes (Seifert et al., 1998; Houston et al., 2002). Accordingly, the agonist-affinities determined in the [³H]mepyramine competition binding studies reflect the agonist-affinities of H₁R in the G-protein-uncoupled state. In fact, the K_i values of agonists **1**, **3**, **12**, **14**, **15**, **19** and **20** at hH₁R and gpH₁R were all higher than the corresponding EC₅₀ values in the GTPase assay (Tables 2 and 4). The K_i value of histamine at hH₁R was 2.3-fold lower than the K_i value of histamine at gpH₁R. Since the amino acids in the histamine-binding H₁R domains are identical in both isoforms (Fig. 3), this difference could point to a better fit of histamine into the G_q-uncoupled hH₁R compared to G_q-uncoupled gpH₁R.

In order to account for the difference in histamine-affinity of H₁R species isoforms, we focused on the comparison of the relative affinities of synthetic agonists at hH₁R and gpH₁R. The relative affinity of the small agonist **3** was similar at hH₁R and gpH₁R, whereas the relative affinities of the 2-phenylhistamines **12**, **14** and **15** and of the histaprodifens **19** and **20** were ~3-7-fold higher at gpH₁R than at hH₁R. These differences fit to the differences in relative agonist potencies observed in the GTPase assay (Table 2). In agreement with the GTPase studies (Table

3), too, the H₁R antagonists triprolidine (**31**), arpromidine (**35**) and BU-E 47 (**36**) all exhibited significantly higher binding affinities at gpH₁R than at hH₁R (Table 4).

We also studied the impact of the F153L/I433V mutation in hH₁R on ligand-affinities. The double mutation significantly decreased the affinity of hH₁R for histamine and 2-(2-thiazolyl)ethanamine (**3**) (Table 4). Similar data were obtained for the comparison of hH₁R and gpH₁R. Additionally, in membranes expressing hH₁R-F153L/I433V, the relative affinities of 2-phenylhistamines and histaprodifens were increased relative to hH₁R, but with the exception of methylhistaprodifen (**19**), those changes were not as marked as for the comparison of hH₁R and gpH₁R. The affinities of triprolidine (**31**), arpromidine (**35**) and guanidine **36** at hH₁R and hH₁R-F153L/I433V were similar.

Potencies and efficacies of H₁R agonists at hH₂R and gpH₂R in the GTPase assay.

The question arose whether H₁R agonists, originally designed for gpH₁R in comparison to gpH₂R, interact differentially with the corresponding human H_xRs. To address this question, we analyzed the effects of H₁R agonists on GTP hydrolysis in Sf9 membranes expressing H₂R-G_{soS} fusion proteins. We examined all H₁R agonists shown in Fig. 1 and listed in Table 2 (**1-23**), but included only those compounds into Table 5 that actually exhibited agonistic activity at H₂Rs. In order to account for the fact that the potency of histamine in the GTPase assay in membranes expressing H₁Rs and H₂Rs differs by almost 10-fold (Tables 2 and 5) (Kelley et al., 2001), we focused on the comparison of relative potencies of H₁R agonists.

2-Methylhistamine (**2**) and 2-(2-thiazolyl)ethanamine (**3**) were strong partial agonists at gpH₂R with moderate (2.3-5-fold) gpH₁R/gpH₂R selectivity. The introduction of a (substituted) phenyl group at position 2 of the imidazole ring greatly reduced the efficacy of H₁R

agonists at gpH₂R and further increased gpH₁R/gpH₂R selectivity in terms of potency. Several 2-phenylhistamines (**11**, **13-15**, **17** and **18**) and histaprodifens **19-21** and **23** were devoid of agonistic activity at gpH₂R-G_{sαS}.

The analysis of histaprodifens at gpH₂R-G_{sαS} revealed the existence of a strong partial H₁R agonist/moderate partial H₂R agonist, N^α-(imidazolyethyl)histaprodifen (**22**) (Tables 2 and 5). The H₂-agonistic activity of this compound can be explained by its structural similarity with guanidines **33-38** (Figs. 1 and 2) that are potent H₂R agonists (Buschauer, 1989; Kelley et al., 2001).

Whereas histamine was similarly potent at stimulating GTP hydrolysis in Sf9 membranes expressing hH₂R-G_{sαS} and gpH₂R-G_{sαS}, 2-methylhistamine (**2**) and 2-(2-thiazolyl)ethanamine (**3**) were significantly less potent agonists at hH₂R-G_{sαS} than at gpH₂R-G_{sαS} and showed greater hH₁R/hH₂R selectivity (8.4-11.2-fold) than gpH₁R/gpH₂R selectivity (2.3-5-fold). If one considers the absolute EC₅₀ values of compound **3** for GTPase activation in membranes expressing hH₁R and hH₂R-G_{sαS}, the selectivity for hH₁R becomes even more striking (75-fold *versus* 23-fold for gpH_xRs). In contrast to compound **3**, another small H₁R agonist, betahistine (**4**), exhibited considerably higher gpH₁R/gpH₂R selectivity (10-fold) than hH₁R/hH₂R selectivity (3.5-fold). Similar to the data obtained for gpH₂R, several 2-phenylhistamines (**11**, **13-15**, **17** and **18**) and histaprodifens (**19-21** and **23**) were devoid of agonistic activity at hH₂R-G_{sαS}. As was the case for gpH_xRs, N^α-(imidazolyethyl)histaprodifen (**22**) was a strong partial hH₁R agonist/moderate partial hH₂R agonist. There were no significant differences in the interaction of histaprodifens at hH₂R-G_{sαS} and gpH₂R-G_{sαS}. Finally, the efficacies of the 2-phenylhistamines **6-9** were significantly lower at hH₂R-G_{sαS} than at gpH₂R-G_{sαS} (Table 6) and therefore in the same order as observed for hH₁R and gpH₁R (Table 2).

Discussion

Pharmacological differences between hH₁R and gpH₁R. hH₁R is an important drug target for treatment of allergic diseases (second-generation H₁R antagonists) and sedation (first-generation H₁R antagonists) (Hill et al., 1997). Preliminary data indicate that pharmacological differences between H₁R species isoforms exist (Chang et al., 1979; Seifert et al., 1994; Elz et al., 2000), but a systematic analysis of this topic has not yet been conducted. Therefore, we studied recombinant hH₁R and gpH₁R with 23 H₁R agonists (**1-23**) (Fig. 1), 22 H₁R antagonists (**24-45**) (Fig. 2) and two H₂R agonists (**46, 47**) under identical experimental conditions, using the GTPase assay (Fig. 5 and Tables 2 and 3) and [³H]mepyramine binding assay (Tables 1 and 4) as read-out.

There were no significant differences between hH₁R and gpH₁R with respect to the potencies and efficacies of small agonists (**1-4**) in the GTPase assay (Fig. 5 and Table 2). However, with respect to bulkier ligands, we found significant differences between hH₁R and gpH₁R. Specifically, H₁R agonists of the 2-phenylhistamine class (**6-17**) and histaprodifen class (**19-23**) were generally more potent and efficacious in the GTPase assay in membranes expressing gpH₁R than in membranes expressing hH₁R (Fig. 5 and Table 2). Additionally, in the binding assay, 2-phenylhistamines and histaprodifens exhibited higher relative affinities for gpH₁R than for hH₁R (Table 4). The differential interaction of 2-phenylhistamine derivatives with gpH₁R and hH₁R is independent of the agonist- or antagonist properties of compounds (compare **17** and **40**, Tables 2 and 3). High constitutive GPCR activity results in high agonist potency and efficacy (Kenakin, 1996; Seifert and Wenzel-Seifert, 2002), but we did not find differences in constitutive activity between hH₁R and gpH₁R studying inverse agonists. Finally, several first-generation H₁R antagonists (**30-32**) and particularly arpromidine-type H₁R

antagonists (**33-38**) showed higher affinities for gpH₁R than for hH₁R. Our data concerning the affinity of ([³H])mepyramine for hH₁R and gpH₁R (Tables 1 and 3) fit very well to previously published data on H₁R species isoforms expressed in native brain (Chang et al., 1979).

Collectively, our data suggest that the ligand-binding site of gpH₁R exhibits a higher conformational flexibility than the ligand-binding site of hH₁R, allowing bulky compounds like 2-phenylhistamines, histaprodifens, mepyramine-type antagonists and guanidines to dock more efficiently into gpH₁R than into hH₁R.

Most of the previous H₁R antagonist development had been conducted with guinea pig models (Hill et al., 1997). Thus, from a therapeutic standpoint, it is fortunate that there are no or only small differences between hH₁R and gpH₁R with respect to commonly used first-generation H₁R antagonists (*e.g.*, **24-28**, **30** and **32**) and second-generation antagonists (**41-45**). However, with regard to the design of H₁R agonists and guanidine-type H₁R antagonists, which are currently used only as experimental tools (Zingel et al., 1995; Hill et al., 1997), the H₁R species isoform is of much greater relevance.

Differences in electrophoretic mobility between hH₁R and gpH₁R. A previous study showed that H₁R isoforms expressed in brain from various species exhibit different migration in SDS-PAGE (Ruat and Schwartz, 1989). These data prompted us to study the electrophoretic mobility of recombinant FLAG epitope-tagged recombinant hH₁R and gpH₁R (Fig. 4). In agreement with the data concerning native H₁R species isoforms, recombinant H₁R species isoforms showed different migration in SDS-PAGE. hH₁R exhibited a moderately higher molecular mass (~76 kDa) than predicted (~56 kDa) (Fukui et al., 1994). hH₁R migrated as mixture of *N*-glycosylated and non-glycosylated protein as assessed by the effect of the inhibitor

of *N*-glycosylation, tunicamycin (Seifert and Wenzel-Seifert, 2001). Recombinant gpH₁R exhibited very different migration in SDS-PAGE than hH₁R, *i.e.*, we detected faint diffuse ~36 kDa- and ~50 kDa bands and intense crisp ~16 kDa- and ~30 kDa bands in Sf9 membranes expressing gpH₁R. In contrast to the results obtained with hH₁R, tunicamycin had no effect on migration of gpH₁R, pointing to different types of *N*-glycosylation in the two H₁R species isoforms. Currently, we do not know the identity of the multiple bands in Sf9 membranes expressing gpH₁R, but highly atypical migration of GPCRs in SDS-PAGE has been repeatedly observed (Grünwald et al., 1996; Kelley et al., 2001; Seifert and Wenzel-Seifert, 2001). In view of the fact that even complex supramolecular structures such as GPCR dimers are preserved in SDS-PAGE (Fukushima et al., 1997; Hebert and Bouvier, 1998; Kelley et al., 2001), it is possible that the different electrophoretic mobilities of hH₁R and gpH₁R reflect different GPCR conformations. The different GPCR conformations may be associated with the specific pharmacological properties of H₁R species isoforms.

Molecular basis for the pharmacological differences between hH₁R and gpH₁R.

Site-directed mutagenesis was successful at identifying the molecular basis for pharmacological differences between species isoforms of H₂R and H₃R (Ligneau et al., 2000; Kelley et al., 2001). We wished to apply the same strategy to H₁R species isoforms. The pharmacological data discussed above indicate that the ligand-binding pocket of gpH₁R is more flexible than the binding pocket of hH₁R. Thus, gpH₁R may possess smaller amino acid substitutions in the ligand-binding domain than hH₁R so that bulkier structures are accommodated more easily in gpH₁R than in hH₁R. In fact, the amino acid substitutions at positions 153 (TM IV) and 433 in hH₁R (TM VI) are bulkier than the corresponding amino acid substitutions in gpH₁R (Phe→Leu

exchange in TM IV and Ile→Val exchange in TM VI, respectively). However, the Phe→Leu exchange in TM IV and the Ile→Val exchange in TM VI only partially explain the differences in agonist-pharmacology between hH₁R and gpH₁R (Tables 2 and 4). Moreover, with respect to the differences in antagonist-pharmacology, the Phe→Leu- and Ile→Val exchanges between hH₁R and gpH₁R are irrelevant (Tables 3 and 4). Thus, additional mutagenesis studies, targeting the top portions of TM II and TM VII are required to elucidate the molecular basis for the pharmacological differences between hH₁R and gpH₁R (Fig. 3).

Although our mutagenesis studies were disappointing in terms of elucidating the molecular basis for the pharmacological differences between hH₁R and gpH₁R, our studies revealed an unexpected role of Phe-153 and Ile-433 in H₁R expression and folding. Specifically, Phe-153→Leu-153- or Ile-433→Val-433 exchange in hH₁R (hH₁R→gpH₁R) resulted in poor receptor expression, low [³H]mepyramine-affinity and functional inactivity (Table 1). Moreover, the mutations grossly altered the electrophoretic mobility of hH₁R (Fig. 4). The double mutation rescued the single mutants in terms of function (Tables 1-4), and it also changed electrophoretic mobility (Fig. 4). These data suggest that the couples Phe-153/Ile-433 or Leu-153/Val-433 are required for a functionally active H₁R. Thus, even conservative amino acid substitutions in TM regions can have profound effects on antagonist-affinity, expression and folding of a GPCR.

Comparison of the effects of H₁R agonists at recombinant and native gpH₁R.

Historically, the guinea pig ileum has been the standard system for the design of H₁R ligands (Zingel et al., 1995; Hill et al., 1997). Therefore, it is important to compare the intact organ data with the results regarding recombinant H₁R. Whereas many highly potent H₂R- and H₃R agonists, *i.e.* ligands ~50-150-fold more potent than histamine, were developed (Hill et al.,

1997), the design of potent H₁R agonists has been a much more difficult task. In fact, the most potent 2-phenylhistamine, 2-(3-trifluoromethylphenyl)histamine (**17**) is only 1.3-fold-, and methylhistaprodifen (**20**) just ~3.5-fold more potent than histamine in the guinea pig ileum (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000) (Table 2).

The expression level of H₁R in the guinea pig ileum is much lower than in the Sf9 cell expression system (Table 1) (Hill et al., 1997). If there had been differences in receptor reserves between the two systems, we would have expected higher agonist efficacies in the recombinant system than in the native system (Hoyer and Boddeke, 1993; Kenakin, 1996). However, the opposite was the case (Table 2) (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000). Thus, we can rule out differences in receptor reserves accounting for the pharmacological differences between the two systems.

All agonists studied with the exception of **1**, **3**, **22** and **23** were more potent at the recombinant gpH₁R than at the native gpH₁R (Table 2). The increase in potency at the recombinant gpH₁R ranged from ~2-fold to almost 20-fold and was most pronounced for the 2-phenylhistamines **7**, **11** and **13**. Several explanations that are not mutually exclusive could account for the potency differences in the two systems. First, there may be substantial penetration barriers for certain agonists to reach the tunica muscularis of the ileum. Second, compounds may accumulate in certain irrelevant cells, *i.e.* epithelial cells and/or, third, may be subject to degradation. These pharmacokinetic factors are very unlikely to be of relevance when assessing the effects of ligands in membrane fragments of insect cells. Fourth, it is possible that differences in gpH₁R glycosylation in insect cells *versus* native tissue contribute to the pharmacological differences in the two systems. Indeed, changes in glycosylation of H₁R have already been shown to alter the pharmacological properties of the GPCR (Mitsubishi and Payan,

1989). Fifth, we studied coupling of H₁Rs to insect cell G_q-proteins (Houston et al., 2002), and the specific type of G_q-protein may have an impact on the pharmacological properties of gpH₁R (Wenzel-Seifert and Seifert, 2000). In contrast to the above-discussed data, the high potency of compounds **22** and **23** in the guinea pig ileum does not fit to the results obtained with recombinant gpH₁R. Additional studies with **22**, **23** and closely related new compounds must be performed to clarify this discrepancy.

Collectively, previous studies on the guinea pig ileum resulted in considerably lower potencies of most H₁R agonists than in the recombinant system. While the high potency of H₂R- and H₃R agonists has not yet been achieved for H₁R agonists, our present study shows that gpH₁R agonists with up to ~12-fold higher potency than histamine exist, provided that the GPCR is analyzed in the GTPase assay using membranes. Thus, future studies on the design of H₁R agonists should be complemented with the recombinant system described herein.

Species-differences in pharmacological properties of H_xRs. H₂R, H₃R and H₄R all exhibit species-differences in their pharmacological properties (Ligneau et al., 2000; Lovenberg et al., 2000; Kelley et al., 2001; Liu et al., 2001). Thus, we were not too surprised to uncover differences in the pharmacological properties of H₁R species isoforms. The species-differences in pharmacological properties of H_xRs extend into H_xR subtype-selectivity of compounds. There are numerous efficacious H₁R agonists of the 2-phenylhistamine- and histaprodifen class with high gpH₁R/gpH₂R selectivity (Tables 2 and 5) (Leschke et al., 1995; Zingel et al., 1995; Elz et al., 2000). However, for the analysis of hH₁R one has to consider the fact that 2-phenylhistamines and histaprodifens possess substantially lower efficacies than histamine (Table 2). Unexpectedly, 2-(2-thiazolyl)ethanamine (**3**), a small agonist with full efficacy at hH₁R,

exhibited a larger hH₁R/hH₂R- than gpH₁R/gpH₂R-selectivity (Tables 2 and 5). Thus, for the analysis of hH₁R with a selective hH₁R agonist, compound **3** may be the ligand of choice. These findings emphasize the importance to study hH_xR isoforms for the development of hH_xR ligands. Future studies will have to answer the question whether the species-differences in pharmacological properties of H_xR reflect species-specific adaptations to as yet unidentified endogenous and/or exogenous H_xR ligands.

Although H₁R and H₂R are structurally quite distinct from each other (only ~40% homology) (Traiffort et al., 1994; Hill et al., 1997), there is a common aspect in the pharmacological properties of these GPCRs, *i.e.* the preferential interaction of bulky agonists with gpH_xR relative to hH_xR. Most notably, arpromidine-derived guanidines represent a class of ligands that exhibit higher affinities for gpH₁R and gpH₂R relative to hH_xR (Tables 3 and 4) (Kelley et al., 2001). Those differences may indicate that gpH_xR in general possess a higher conformational flexibility than hH_xR.

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Footnotes

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Figure legends

Fig. 1. Structures of H₁R agonists. **1**, Histamine; **2-4**, small histamine-derived agonists; **5-17**, 2-phenylhistamine derivatives; **18**, thioether compound related to **5-17**; **18-23**, histaprodifens. For agonist names, see Table 2.

Fig. 2. Structures of H₁R antagonists. **24-32**, First-generation H₁R antagonists; **33-39**, arpromidine (**35**)-derived dual H₂R agonists/H₁R antagonists; **40**, H₁R antagonist derived from 2-phenylhistamines; **41-45**, second-generation H₁R antagonists. For antagonist names, see Table 3.

Fig. 3. Snake plot of hH₁R showing the relative positions and topology of amino acid residues in the TM domains, putative agonist- and antagonist binding sites and differences with respect to the gpH₁R. Fig. 3 considers results of the substituted-cysteine accessibility method by which the dopamine D₂-receptor binding site was mapped (Ballesteros et al., 2001). *Red circles*, amino acids predicted to face the interior of the TM helix bundle based on corresponding positions in bovine rhodopsin (Palczewski et al., 2000). *Blue circles*, amino acids predicted to face lipid. *Red letters*, the corresponding amino acids in the dopamine D₂-receptor are water-accessible and protected by ligand binding in substituted-cysteine accessibility experiments (Ballesteros et al., 2001). *Magenta letters*, amino acids that are water-accessible but not protected by ligand binding in substituted-cysteine accessibility experiments. *Red shading*, histamine binding site residues according to site-directed mutagenesis data (Leurs et al., 1994, 1995; Ohta et al., 1994; Nonaka et al., 1998). *Green shading*, amino acids of the H₁R antagonist binding site based on mutagenesis studies (Ohta et al., 1994; Nonaka et al., 1998; Wieland et al., 1999). *Yellow shading*, amino acids that are essential for both histamine and H₁R antagonist

binding (Wieland et al., 1999). *Orange shading*, additional amino acid residues of the histaprodifen binding site based on molecular modeling approaches (Elz et al., 2000). Note that Lys-191 does not interact with histaprodifens. *Blue shading*, amino acids targeted by site-directed mutagenesis in the present paper. *Grey shading*, other amino acid residues different between hH₁R and gpH₁R. *Brown line*, conserved disulfide bridge between Cys-100 and Cys-180. Numbers designate the first and last amino acids, respectively, of each TM domain.

Fig. 4. Analysis of the expression of H₁R constructs in Sf9 cell membranes. **A**, Sf9 cells were cultured in the absence (-) of tunicamycin (Tuna.) or in the presence (+) of tunicamycin (10 µg/ml). Sf9 cell membranes expressing hH₁R or gpH₁R were separated by SDS-PAGE on a gel that contained 10% (w/v) acrylamide. Membranes were probed with the anti-FLAG Ig (M1 antibody). Each membrane preparation was analyzed in two different amounts (25 µg and 50 µg of protein per lane, respectively). **B**, Sf9 cell membranes expressing hH₁R-F153L, hH₁R-I433V, hH₁R-F153L/I433V and hH₁R were separated by SDS-PAGE on a gel that contained 10% (w/v) acrylamide. Membranes (75 µg of protein/lane each) were probed with the anti-FLAG Ig. *Numbers on the left of the immunoblots in A and B indicate molecular masses of marker proteins.* The horseradish peroxidase-reacted Immobilon P membranes of representative gels are shown. Similar results were obtained with 3-6 other membrane preparations of hH₁R constructs. Expression levels of H₁R constructs in terms of [³H]mepyramine saturation binding are given in Table 1.

Fig. 5. Relations between the potencies of H₁R agonists at hH₁R and gpH₁R expressed in Sf9 membranes in the GTPase assay. pEC₅₀ values of agonists at hH₁R and gpH₁R were

derived from EC_{50} values shown in Table 1 and analyzed by linear regression. Solid lines represent the actual correlations obtained. Dashed lines represent the 95% confidence intervals of the correlations. The straight dotted lines represent the theoretical correlations describing pharmacological identity between the H_1R species isoforms. The theoretical curves have a slope of 1.00. **A**, Correlation of the potencies of small agonists (**1-4**) at hH_1R versus gpH_1R . Slope, 0.74 ± 0.03 ; r^2 , 0.99; $p < 0.001$ (significant). **B**, Correlation of potencies of 2-phenylhistamines (**6-18**) at hH_1R versus gpH_1R . Slope, 0.96 ± 0.07 ; r^2 , 0.95; $p < 0.001$ (significant). **C**, Correlation of potencies of histaprodifens (**19-23**) at hH_1R versus gpH_1R . Slope, 0.54 ± 0.08 ; r^2 , 0.96; $p < 0.001$ (significant).

Table 1. [³H]Mepyramine saturation binding in Sf9 membranes expressing hH₁R, gpH₁R, hH₁R-F153L, hH₁R-I433V and hH₁R-F153L/I433V

H₁R construct	K_d (nM)	B_{max} (pmol/mg)
hH ₁ R	4.49 ± 0.35	5.85 ± 1.67
gpH ₁ R	2.53 ± 0.23*	3.94 ± 0.83
hH ₁ R-F153L	37.8 ± 15.8*	0.88 ± 0.18*
hH ₁ R-I433V	53.7 ± 26.2*	1.13 ± 0.03*
hH ₁ R-F153L/I433V	4.33 ± 0.40	15.7 ± 6.4*

Sf9 membranes expressing various H₁R constructs (+ RGS4 or GAIP) were incubated with 0.2-100 nM [³H]mepyramine as appropriate according to the protocol described in *Methods*.

Nonspecific binding was determined in the presence of 10 μM mepyramine and was subtracted from total [³H]mepyramine binding. Binding data were analyzed by non-linear regression and were best fit to monophasic saturation curves. Data shown are the means ± SD of 3-4 membrane preparations analyzed in triplicates each. *, *p* < 0.05 for comparison of hH₁R *versus* other H₁R constructs.

Table 2. Potencies and efficacies of H₁R- and H₂R agonists in the GTPase assay in Sf9 membranes expressing hH₁R, gpH₁R and hH₁R-F153L/I433V: Comparison with the guinea pig ileum

Cpd.	agonist	hH ₁ R			gpH ₁ R			hH ₁ R-F153L/I433V				gp-ileum		
		EC ₅₀ (μM)	rel. pot.	<i>E</i> _{max}	EC ₅₀ (μM)	rel. pot.	<i>E</i> _{max}	pot. rat. gp/h	EC ₅₀ (μM)	rel. pot.	<i>E</i> _{max}	pot. rat. m/h	rel. pot.	pot. rat. rec/il
1	Histamine	0.184 ± 0.094	100	1.00	0.220 ± 0.047	100	1.00	0.84	0.163 ± 0.029	100	1.00	1.13	100	1.00
2	2-Methylhistamine	0.837 ± 0.110	22.0	0.98 ± 0.03	0.708 ± 0.181	31.1	0.97 ± 0.04	0.87	n. d.	n. d.	n. d.	n. d.	14.0	2.22
3	2-(2-Thiazolyl)-ethanamine	0.440 ± 0.116	41.8	0.97 ± 0.13	0.433 ± 0.133	50.8	1.00 ± 0.08	1.02	0.373 ± 0.076	43.7	1.08 ± 0.05*	1.17	45.0	1.13
4	Betahistine	1.438 ± 0.368	12.8	0.86 ± 0.10	0.963 ± 0.277	22.8	0.86 ± 0.09	1.49	n. d.	n. d.	n. d.	n. d.	8.0	2.85
5	2-Benzylhistamine	6.518 ± 0.482	2.80	0.75 ± 0.10	5.234 ± 1.432	4.20	0.88 ± 0.11	1.24	3.130 ± 0.060*	5.21	0.74 ± 0.01	2.08	2.5	1.68
6	2-Phenylhistamine	0.877 ± 0.201	21.0	0.79 ± 0.07	0.160 ± 0.010*	137.5	0.98 ± 0.09*	5.48	0.638 ± 0.100	25.5	0.91 ± 0.06	1.37	31.0	4.44
7	2-(3-Methylphenyl)-histamine	1.088 ± 0.240	17.0	0.77 ± 0.11	0.134 ± 0.065*	164.2	0.89 ± 0.05	8.06	n. d.	n. d.	n. d.	n. d.	14.7	11.2
8	2-(2-Fluorophenyl)-histamine	2.783 ± 1.092	6.6	0.71 ± 0.04	0.766 ± 0.231*	28.9	0.88 ± 0.14*	3.66	n. d.	n. d.	n. d.	n. d.	5.8	4.98
9	2-(3-Fluorophenyl)-histamine	0.683 ± 0.303	26.9	0.75 ± 0.04	0.073 ± 0.022*	301.4	0.89 ± 0.08*	9.39	0.263 ± 0.079	62.0	0.85 ± 0.09	2.60	85.0	3.55
10	2-(4-Fluorophenyl)-histamine	3.220 ± 0.569	5.5	0.60 ± 0.12	0.587 ± 0.127*	37.5	0.80 ± 0.07*	5.65	n. d.	n. d.	n. d.	n. d.	12.5	3.00
11	2-(3,5-Difluorophenyl)histamine	0.765 ± 0.133	24.0	0.59 ± 0.10	0.132 ± 0.029*	166.7	0.83 ± 0.11*	5.80	n. d.	n. d.	n. d.	n. d.	15.0	11.1
12	2-(3-Chlorophenyl)-	0.434 ± 0.128	42.4	0.71 ± 0.05	0.081 ± 0.026*	271.6	0.89 ± 0.10*	5.35	0.160 ± 0.056*	101.9	0.81 ± 0.08*	2.71	96.0	2.83

	histamine													
13	2-(4-Chlorophenyl)- histamine	16.440 ± 3.880	1.1	0.54 ± 0.11	2.313 ± 0.788*	9.5	0.56 ± 0.11	7.11	n. d.	n. d.	n. d.	n. d.	0.5	19.0
14	2-(3-Bromophenyl)- histamine	0.210 ± 0.065	87.6	0.73 ± 0.01	0.053 ± 0.002*	415.1	0.95 ± 0.11*	3.96	0.143 ± 0.032	114.0	0.75 ± 0.05	1.47	112	3.71
15	2-(3-Iodophenyl)- histamine	0.220 ± 0.118	83.6	0.70 ± 0.08	0.040 ± 0.021*	550.0	0.86 ± 0.05*	5.50	0.133 ± 0.051	122.6	0.70 ± 0.09	1.65	96.0	5.73
16	2-(3-Methoxyphenyl)- histamine	0.262 ± 0.100	70.2	0.74 ± 0.05	0.060 ± 0.017*	366.7	1.00 ± 0.09*	4.37	n. d.	n. d.	n. d.	n. d.	42.1	8.71
17	2-(3-Trifluoromethyl- phenyl)histamine	0.243 ± 0.091	41.8	0.74 ± 0.04	0.057 ± 0.023*	386.0	0.91 ± 0.12*	4.26	n. d.	n. d.	n. d.	n. d.	129	2.99
18	2-(4-Methylphenylthio- methyl)histamine	2.54 ± 0.36	7.2	0.50 ± 0.05	0.77 ± 0.18*	29.9	0.80 ± 0.11*	3.18	n. d.	n. d.	n. d.	n. d.	12.7	2.35
19	Methylhistaprodifen	0.068 ± 0.018	270.5	0.77 ± 0.10	0.019 ± 0.006*	1157.8	0.87 ± 0.06	3.57	0.040 ± 0.011	407.5	0.88 ± 0.06	1.70	343	3.38
20	Dimethylhistaprodifen	0.100 ± 0.026	184.0	0.64 ± 0.09	0.027 ± 0.012*	814.8	0.84 ± 0.06*	3.70					242	3.37
21	Pyrrolidinohistaprodifen	0.293 ± 0.120	62.8	0.19 ± 0.02	0.044 ± 0.013*	500.0	0.40 ± 0.03*	6.66	0.258 ± 0.217	63.2	0.34 ± 0.05	1.14	67.0	7.46
22	N ⁶ -(Imidazolylethyl)- histaprodifen	0.238 ± 0.138	77.3	0.84 ± 0.08	0.050 ± 0.025*	440.0	0.89 ± 0.09	4.76	n. d.	n. d.	n. d.	n. d.	3630	0.12
23	Dimeric histaprodifen	0.653 ± 0.210	28.2	0.65 ± 0.11	0.066 ± 0.033*	333.3	0.92 ± 0.08*	9.85	n. d.	n. d.	n. d.	n. d.	1680	0.20
46	Amthamine	-	-	0.01 ± 0.02	150 ± 56	-	0.14 ± 0.05*	-	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
47	Dimaprit	-	-	0.06 ± 0.04	-	-	0.06 ± 0.03	-	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

Steady-state GTPase activity in Sf9 membranes expressing H₁R constructs (+ RGS4 or GAIP) was determined as described in *Methods*. Reaction mixtures contained H_xR ligands at concentrations from 1 nM - 1 mM as appropriate to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Typical basal GTPase activities ranged between 1.5-2.5 pmol/mg/min, and the maximum stimulatory effect of histamine (10 μM) amounted to 125-175% above basal (Houston et al., 2002). The efficacy (E_{max}) of histamine was determined by non-linear regression and was set 1.00. The E_{max} values of other agonists were referred to this value. Data shown are the means ± SD of 5-8 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH₁R *versus* other H₁R constructs. The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value. We also calculated the ratio of the EC₅₀ values of H₁R agonists for hH₁R and gpH₁R (pot. rat. gp/h) and the ratio of the EC₅₀ values of H₁R agonists for hH₁R and hH₁R-F153L/I433V (pot. rat. m/h). Table 2 also shows the relative potencies of H₁R agonists in the standard system for the analysis of the H₁R, the guinea pig ileum. The EC₅₀ of histamine in this system is ~0.15-0.20 μM (Zingel et al., 1990; Elz et al., 2000). The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value. Finally, we calculated the ratio of the relative potency of H₁R agonists at the native gpH₁R expressed in the ileum and recombinant gpH₁R expressed in Sf9 membranes (pot. rat. rec/il). The relative potencies of compounds **2-23** in the guinea pig ileum were calculated from isotonicity recorded cumulative concentration/response curves on whole segments of ileum (preload 0.5 g) in the continuous presence of atropine (100 nM) as described (Elz et al., 2000). gp-ileum, guinea pig ileum; n. d., not determined; -, not applicable because stimulatory effects of agonists were too small; Cpd., compound.

Table 3. Potencies of H₁R antagonists in the GTPase assay in Sf9 membranes expressing hH₁R, gpH₁R and hH₁R-F153L/I433V

Cpd.	antagonist	K_B hH ₁ R (nM)	K_B gpH ₁ R (nM)	pot. rat. gpH ₁ R/hH ₁ R	hH ₁ R-F153L/I433V	pot. rat. mut./hH ₁ R
24	Promazine	1.08 ± 0.11	1.30 ± 0.19	0.83	n. d.	n. d.
25	Chlorpromazine	2.55 ± 0.54	2.85 ± 1.34	0.89	n. d.	n. d.
26	Mianserin	2.13 ± 1.17	3.16 ± 1.88	0.67	n. d.	n. d.
27	Cyproheptadine	1.92 ± 0.13	2.64 ± 0.94	0.73	n. d.	n. d.
28	Diphenhydramine	15.5 ± 1.77	16.4 ± 1.99	0.95	n. d.	n. d.
(R)-29	(R)-(-)-Dimethindene	2.68 ± 1.52	2.97 ± 1.49	0.90	n. d.	n. d.
(S)-29	(S)-(+)-Dimethindene	113.2 ± 30.9	94.5 ± 38.8	1.20	n. d.	n. d.
30	Mepyramine	5.67 ± 0.88	2.30 ± 0.52*	2.47	5.76 ± 1.10	0.99
31	Triprolidine	4.37 ± 1.61	1.73 ± 0.32*	2.52	n. d.	n. d.
32	(+)-Chlorpheniramine	9.85 ± 2.32	4.97 ± 1.37*	1.98	n. d.	n. d.
33	BU-E 42	554 ± 36.8	98.6 ± 19.7*	5.62	698 ± 31.8*	0.80
34	BU-E 43	332 ± 74.0	142 ± 37.7*	2.34	n. d.	n. d.
35	Arpromidine	332 ± 89.6	48.9 ± 12.9*	6.79	465 ± 88.3	0.72
36	BU-E 47	724 ± 180	80.0 ± 16.8*	9.05	477 ± 61.0*	1.51

37	BU-E 48	543 ± 198	116 ± 31.0*	4.68	528 ± 29.7	1.03
38	BU-E 75	499 ± 122	131 ± 10.4*	3.81	1180 ± 64*	0.42
39	D281	562 ± 123	352 ± 79	1.60	n. d.	n. d.
40	<i>N</i> -{2-[2-(3-Trifluoromethylphenyl)-1 <i>H</i> -imidazol-4-yl]ethyl}piperidine	1570 ± 148	454 ± 155*	3.46	2310 ± 210*	0.68
41	Terfenadine	28.1 ± 4.97	38.5 ± 6.08	0.73	n. d.	n. d.
42	Fexofenadine	226 ± 50.2	258 ± 61.0	0.88	n. d.	n. d.
43	Astemizole	10.4 ± 1.42	12.7 ± 3.25	0.82	n. d.	n. d.
44	Azelastine	1.71 ± 0.16	1.89 ± 0.17	0.90	n. d.	n. d.
45	Ketotifen	0.96 ± 0.03	1.09 ± 0.30	0.88	n. d.	n. d.

Steady-state GTPase activity in Sf9 membranes expressing H₁R constructs (+ RGS4 or GAIP) was determined as described in *Methods*. Reaction mixtures contained 1 μM histamine and H₁R antagonists at concentrations from 1 nM - 100 μM as appropriate to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Typical GTPase activities in the presence of 1 μM histamine between 2.5-3.0 pmol/mg/min. Data shown are the means ± SD of 3-5 experiments performed in duplicates each. * *p* < 0.05 for comparison of hH₁R *versus* other H₁R constructs. We also calculated the ratio of the *K_B* values for hH₁R and gpH₁R (pot. rat. gpH₁R/hH₁R) and the ratio of the *K_B* values for hH₁R and hH₁R-F153L/I433V (pot. rat. mut./hH₁R). n. d., not determined. Cpd., compound.

Table 4. Agonist- and antagonist-affinities of hH₁R, gpH₁R and hH₁R-F153L/I433V expressed in Sf9 membranes in the [³H]mepyramine competition binding assay

Cpd.	ligand	hH ₁ R		gpH ₁ R			hH ₁ R-F153L/I433V		
		K _i	rel. aff.	K _i	rel. aff.	aff. rat. gp/h	K _i	rel. aff.	aff. rat. m/h
1	Histamine	2.06 ± 0.18 μM	100	4.65 ± 0.26 μM*	100	0.44	3.30 ± 0.60 μM*	100	0.62
3	2-(2-Thiazolyl)ethanamine	4.60 ± 1.93 μM	44.8	8.49 ± 3.53 μM	54.8	0.54	12.7 ± 1.70 μM*	26	0.36
12	2-(3-Chlorophenyl)histamine	1.78 ± 0.30 μM	115.7	0.60 ± 0.17 μM*	775.0	2.97	1.53 ± 0.18 μM	215.7	1.16
14	2-(3-Bromophenyl)histamine	2.22 ± 0.30 μM	107.8	0.70 ± 0.10 μM*	668.1	3.19	1.43 ± 0.24 μM	230.8	1.55
15	2-(3-Iodophenyl)histamine	1.76 ± 0.24 μM	117.1	0.61 ± 0.14 μM*	759.8	2.88	1.38 ± 0.02 μM	239.1	1.28
19	Methylhistaprodifen	0.37 ± 0.07 μM	552.3	0.29 ± 0.06 μM	1603.4	1.29	0.24 ± 0.04 μM*	1375	1.54
20	Dimethylhistaprodifen	0.40 ± 0.06 μM	509.9	0.31 ± 0.08 μM	1480.9	1.29	0.36 ± 0.02 μM	916.7	1.11
31	Triprolidine	3.01 ± 0.54 nM	-	1.15 ± 0.02 nM*	-	2.62	2.88 ± 0.15 nM	-	1.05
35	Arpromidine	353 ± 71 nM	-	33.3 ± 10.1 nM*	-	10.6	282 ± 57 nM	-	1.25
36	BU-E 47	255 ± 68 nM	-	53.9 ± 14.8 nM*	-	4.73	321 ± 56 nM	-	0.79

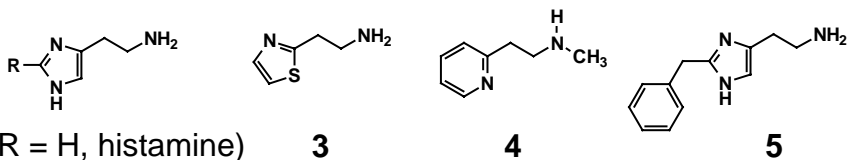
[³H]Mepyramine competition binding in Sf9 membranes expressing H₁R constructs (+ RGS4 or GAIP) was determined as described in *Methods*. Reaction mixtures contained 2 nM [³H]mepyramine and unlabeled H₁R ligands at concentrations of 0.1 nM - 10 mM as appropriate to generate saturated competition curves. Data were analyzed by non-linear regression and were best fit to one-site (monophasic) competition curves. Data shown are the means ± SD of 3-5 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH₁R *versus* other H₁R constructs. The relative affinity of histamine (rel. aff.) was set 100, and the affinities of other agonists were referred to this value. We also calculated the ratio of the K_i values for hH₁R and gpH₁R (aff. rat. gp/h) and the ratio of the K_i values for hH₁R and hH₁R-F153L/I433V (aff. rat. m/h). -, not applicable. Cpd., compound.

Table 5. Potencies and efficacies of H₁R agonists in the GTPase assay in Sf9 membranes expressing hH₂R-G_{soS} and gpH₂R-G_{soS}

Cpd.	Agonist	hH ₂ R				gpH ₂ R				
		EC ₅₀ (μM)	rel. pot.	<i>E</i> _{max}	rel. pot. rat. hH ₁ R/hH ₂ R	EC ₅₀ (μM)	rel. pot.	<i>E</i> _{max}	rel. pot. rat. gpH ₁ R/ gpH ₂ R	pot. rat. gpH ₂ R/ hH ₂ R
1	Histamine	1.26 ± 0.25	100	1.00	1.00	1.20 ± 0.24	100	1.00	1.00	1.05
2	2-Methylhistamine	47.9 ± 18.5*	2.63	0.93 ± 0.05	8.36	8.82 ± 1.48	13.6	0.87 ± 0.06	2.29	5.43
3	2-(2-Thiazolyl)ethanamine	33.9 ± 9.21*	3.72	0.91 ± 0.11	11.2	11.8 ± 1.12	10.2	0.90 ± 0.16	4.98	2.87
4	Betahistine	33.6 ± 7.23	3.75	0.73 ± 0.07	3.41	51.4 ± 8.3	2.33	0.73 ± 0.06	9.79	0.65
5	2-Benzylhistamine	27.9 ± 3.33	4.52	0.37 ± 0.04*	0.62	28.5 ± 15.6	4.21	0.54 ± 0.04	1.00	0.98
6	2-Phenylhistamine	38.7 ± 6.12	3.26	0.20 ± 0.03*	6.44	59.6 ± 20.5	2.01	0.38 ± 0.05	68.4	0.65
7	2-(3-Methylphenyl)histamine	-	-	0.06 ± 0.03*	-	12.1 ± 4.5	9.92	0.16 ± 0.04	16.6	-
8	2-(2-Fluorophenyl)histamine	63.8 ± 22.6	1.98	0.23 ± 0.03*	3.33	89.9 ± 6.1	1.33	0.43 ± 0.02	21.7	0.71
9	2-(3-Fluorophenyl)histamine	25.6 ± 10.6	4.92	0.17 ± 0.04*	5.47	42.3 ± 9.6	2.84	0.28 ± 0.02	227	0.61
10	2-(4-Fluorophenyl)histamine	11.5 ± 3.7	10.6	0.10 ± 0.02	0.52	10.8 ± 4.3	11.1	0.14 ± 0.05	13.2	1.06
12	2-(3-Chlorophenyl)histamine	-	-	0.08 ± 0.04	-	41.4 ± 14.8	2.90	0.15 ± 0.03	93.7	-
16	2-(3-Methoxyphenyl)- histamine	-	-	0.08 ± 0.05	-	90.4 ± 23.8	1.32	0.13 ± 0.04	278	-

22	<i>N</i> ^α -(Imidazolylethyl)- histaprodifen	0.570 ± 0.133	221	0.39 ± 0.06	0.35	0.470 ± 0.085	255	0.46 ± 0.05	1.73	1.21
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Steady-state GTPase activity in Sf9 membranes expressing H₂R-G_{sα} fusion proteins was determined as described in *Methods*. Reaction mixtures contained H₁R ligands at concentrations from 10 nM - 1 mM as appropriate to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Typical basal GTPase activities ranged between 1.0-2.0 pmol/mg/min, and the maximum stimulatory effect of histamine (100 μM) amounted to 200-300% above basal (Kelley et al., 2001). The efficacy (E_{max}) of histamine was determined by non-linear regression and was set 1.00. The E_{max} values of other H₁R agonists were referred to this value. Data shown are the means ± SD of 3-4 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH₂R *versus* gpH₂R. The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value. We also calculated the ratio of the relative potencies of H₁R agonists at hH₁R (taken from Table 2) and hH₂R-G_{sαS} (rel. pot. rat. hH₁R/hH₂R) and the ratio of the relative potencies of H₁R agonists at gpH₁R (taken from Table 2) and gpH₂R-G_{sαS} (rel. pot. rat. gpH₁R/gpH₂R). Additionally, we calculated the ratio of the EC₅₀ values of H₁R agonists for hH₂R-G_{sαS} and gpH₂R-G_{sαS} (pot. rat. gpH₂R/hH₂R). Data for compounds **1** and **4** were taken from Kelley et al. (2001). Cpd., compound; -, not applicable because stimulatory effects of agonists were too small. Compounds 11, **13-15**, 17-21 αδ 23 ατ χονχεντρατιονσ φρομ 10 μM - 1 mM were devoid of any stimulatory effect on GTPase activity in membranes expressing hH₂R-G_{sαS} and gpH₂R-G_{sαS} and, therefore, not shown in the Table.

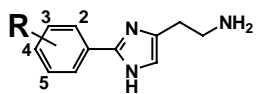


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2 (R = CH₃)

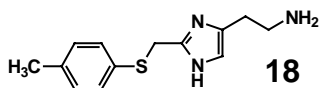
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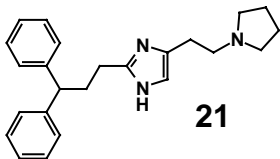
5



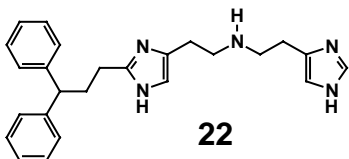
no.	R
6	H
7	3-CH ₃
8	2-F
9	3-F
10	4-F
11	3,5-F ₂
12	3-Cl
13	4-Cl
14	3-Br
15	3-I
16	3-OCH ₃
17	3-CF ₃



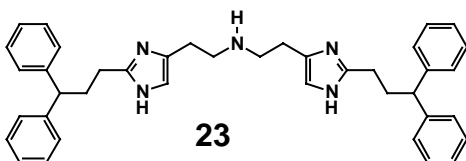
18



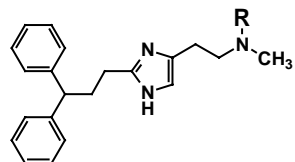
21



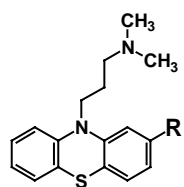
22



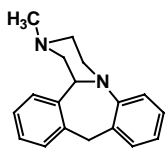
23



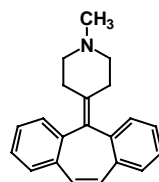
no.	R
19	H
20	CH ₃



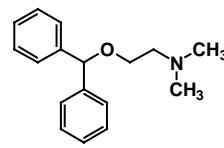
24 (R = H)
25 (R = Cl)



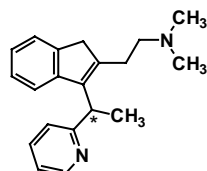
26



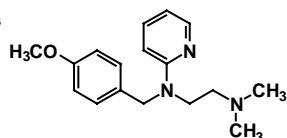
27



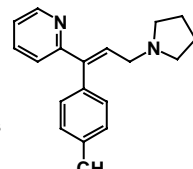
28



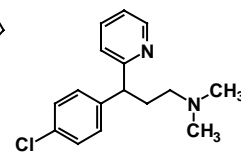
(R)-(-)-29
(S)-(+)-29



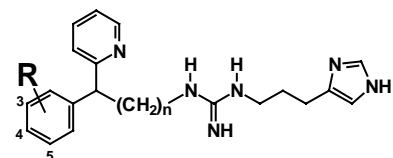
30



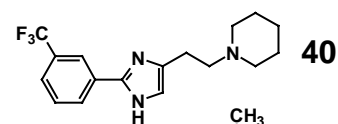
31



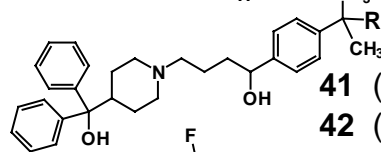
32



no.	n	R
33	2	H
34	3	H
35	2	4-F
36	2	4-Cl
37	2	4-Br
38	2	3,4-Cl ₂

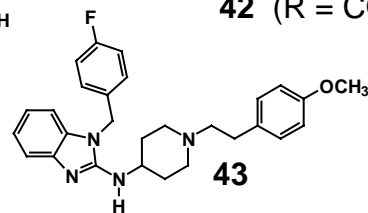


40

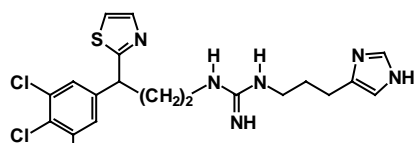


41 (R = CH₃)

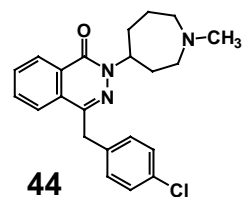
42 (R = COOH)



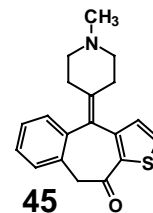
43



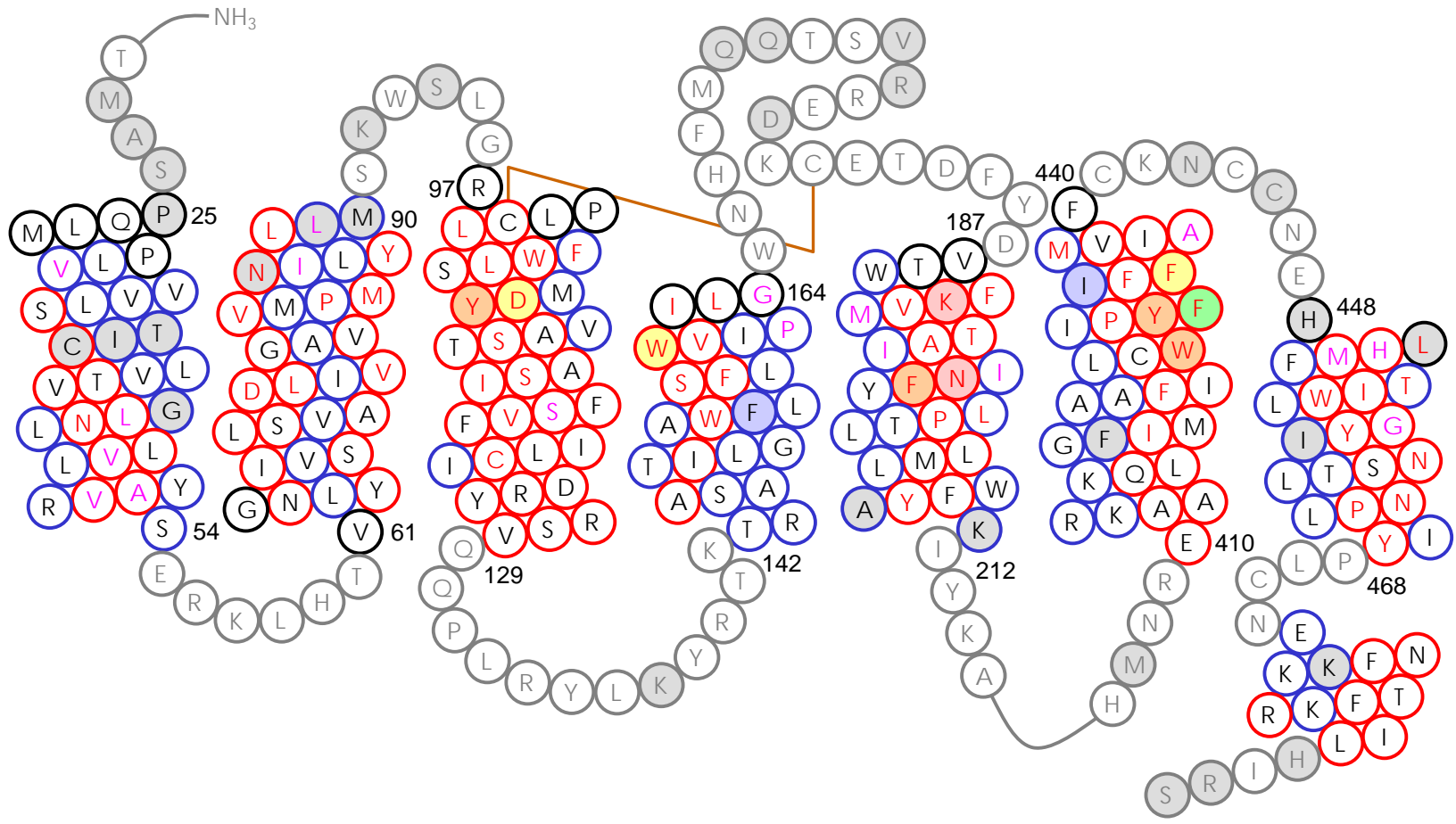
39

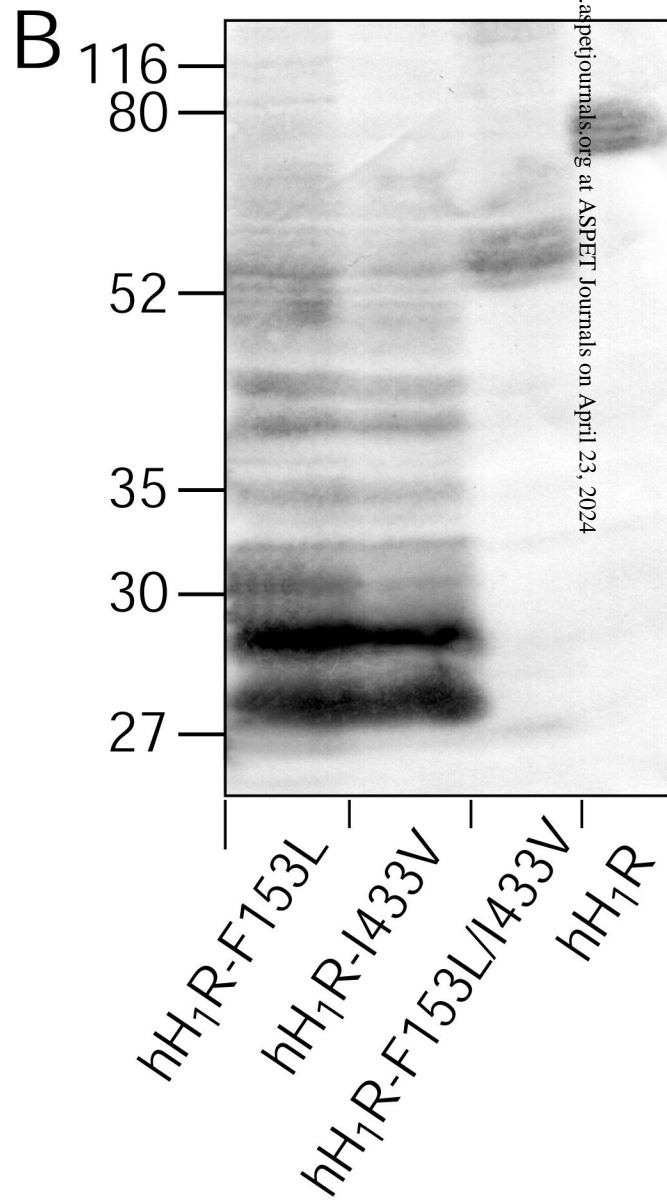
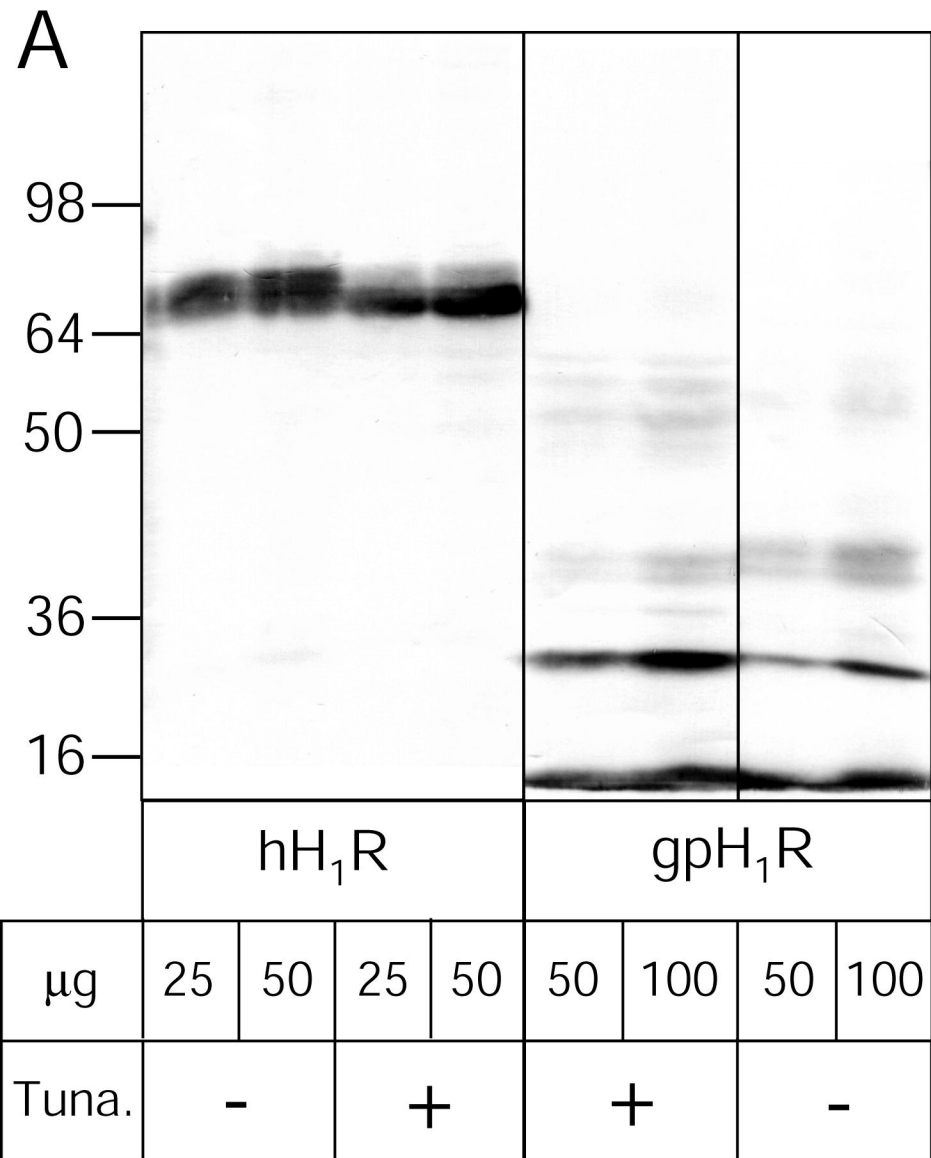


44

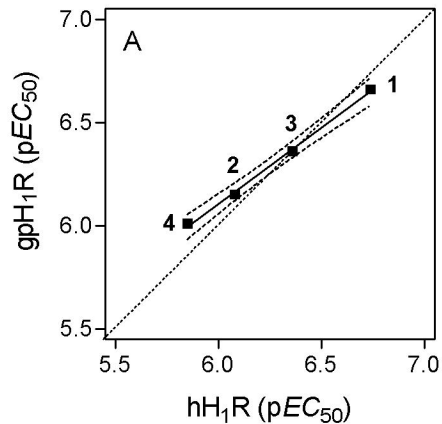


45

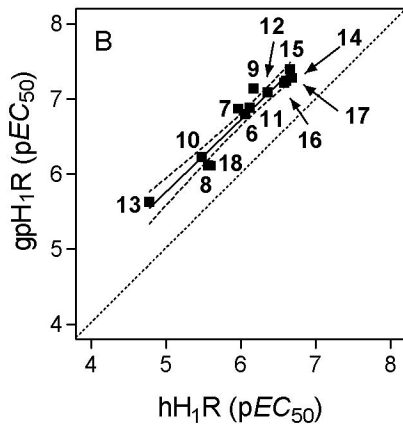




small agonists



2-phenylhistamines



histaprodifens

