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Title Page

Probing ligand-specific histamine H_1- and H_2-receptor conformations with N^G-acylated imidazolylpropylguanidines

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Running Title Page

Running title: Ligand-specific histamine receptor conformations

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Abbreviations used: AC, adenylyl cyclase; AIPG, N^G-acylated imidazolylpropylguanidine; AMT, amthamine; ARP, arpromidine; DIM, dimaprit; GPCR, G-protein-coupled receptor; gpH_1R, guinea pig histamine H_1-receptor; gpH_2R, guinea pig histamine H_2-receptor; gpH_2R-$G_{s\alpha S}$, fusion protein of the guinea pig histamine H_2-receptor and the short splice variant of $G_{s\alpha}$; GTPγS, guanosine 5'-[γ-thio]triphosphate; hH_1R, human histamine H_1-receptor; hH_2R, human histamine H_2-receptor; hH_2R-$G_{s\alpha S}$, fusion protein of the human histamine H_2-receptor and the short splice variant of $G_{s\alpha}$; HIS, histamine; IMP, impromidine; RGS protein, regulator of G-protein signaling; rH_2R, rat histamine H_2-receptor; TM, transmembrane domain.

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Abstract

Impromidine (IMP) and arpromidine (ARP)-derived guanidines are more potent and efficacious guinea pig (gp) histamine H_2-receptor (gpH_2R)- than human (h) H_2R agonists and histamine H_1-receptor (H_1R) antagonists with preference for hH_1R relative to gpH_1R. We examined N^G-acylated imidazolylpropylguanidines (AIPGs) which are less basic than guanidines at hH_2R, gpH_2R, rat H_2R (rH_2R), hH_1R and gpH_1R expressed in Sf9 cells as probes for ligand-specific receptor conformations. AIPGs were similarly potent H_2R agonists as the corresponding guanidines IMP and ARP, respectively. Exchange of pyridyl in ARP against phenyl increased AIPG potency ten-fold, yielding the most potent agonists at the hH_2R-$G_{s\alpha}$ fusion protein and gpH_2R-$G_{s\alpha}$ identified so far. Some AIPGs were similarly potent and efficacious at hH_2R-$G_{s\alpha}$ and gpH_2R-$G_{s\alpha}$. AIPGs stabilized the ternary complex in hH_2R-$G_{s\alpha}$ and gpH_2R-$G_{s\alpha}$ differently than the corresponding guanidines. Guanidines, AIPGs and small H_2R agonists exhibited distinct agonist properties at hH_2R, gpH_2R and rH_2R measuring adenylyl cyclase activity. In contrast to ARP and IMP, AIPGs were partial H_1R agonists exhibiting higher efficacies at hH_1R than at gpH_1R. This is remarkable since so far, all bulky H_1R agonists exhibited higher efficacies at gpH_1R than at hH_1R. Collectively, our data suggest that AIPGs stabilize different active conformations in hH_2R, gpH_2R and rH_2R than guanidines and that in contrast to guanidines, AIPGs are capable of stabilizing a partially active state of hH_1R.

Introduction

HIS exerts its biological effects through the H_1R, H_2R, H_3R and H_4R, respectively (Hill et al., 1997; Hough, 2001). We are particularly interested in the H_1R and H_2R (Klinker et al., 1995; Seifert et al., 2003; Dove et al., 2004). The H_1R couples to G_q-proteins mediating phospholipase C activation, and the H_2R couples to G_s-proteins mediating AC activation (Hill et al., 1997). In some systems, the H_2R also couples to G_q-proteins (Kühn et al., 1996; Leopoldt et al., 1997). We established expression systems for the H_1R and H_2R in Sf9 insect cells (Houston et al., 2002). Sf9 cells can be cultured in large amounts and yield high GPCR expression levels. In Sf9 cell membranes GPCR/G-protein coupling can be measured with high sensitivity using the steady-state GTPase activity. An advantage of the GTPase assay is that it assesses GPCR/G-protein coupling at a proximal level, avoiding potential bias introduced by assessing more downstream events such as effector activation or changes in gene expression. In case of H_1R, coupling of the GPCR to insect cell G_q-proteins is determined, using RGS proteins as signal enhancers for GTPase activity (Houston et al., 2002; Seifert et al., 2003). In case of H_2R, fusion proteins of GPCR and mammalian $G_{s\alpha}$ proteins are used (Kelley et al., 2001; Wenzel-Seifert et al., 2001). GPCR-$G_{s\alpha}$ fusion proteins ensure defined 1 : 1 stoichiometry of the coupling partners and their efficient interaction (Seifert et al., 1999a). By measuring GTP hydrolysis, potencies and efficacies of H_2R agonists are assessed in an expression level-independent manner (Seifert et al., 1999a; Kelley et al., 2001; Wenzel-Seifert et al., 2001). The H_2R is differently glycosylated in mammalian and insect cells, but glycosylation does not affect the pharmacological GPCR properties (Fukushima et al., 1995; Houston et al., 2002). Moreover, the pharmacological properties of H_1R and H_2R expressed in mammalian and insect cells are similar, rendering the latter system a useful model for extensive pharmacological studies (Kühn et al., 1996; Beukers et al., 1997; Leopoldt et al., 1997; Kelley et al., 2001; Seifert et al., 2003).

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H_2R agonists are divided into two classes. The first class comprises small molecules related to HIS (**1**), most importantly AMT (**2**) and DIM (**3**) (Fig. 1). The amino group of HIS forms an ionic interaction with Asp-98 in TM3, and the imidazole ring interacts with Tyr-182 and Asp-186 in TM5 (Gantz et al., 1992; Nederkoorn et al., 1994). Small H_2R agonists activate hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ with similar potency and efficacy (Kelley et al., 2001). The second class of H_2R agonists comprises long-chained and more bulky molecules, IMP (**4**) and ARP (**6**) being the prototypes (Durant et al., 1978; Buschauer, 1989). The guanidino group and the imidazolylpropyl moieties of IMP and ARP form similar interactions with H_2R as the amino group and imidazole groups of HIS, respectively (Kelley et al., 2001). Additionally, the 2-(5-methylimidazol-4-ylmethylthio)ethyl moiety of IMP and the 3-(4-fluorophenyl)-3-pyridylpropyl substituents of ARP interact with a pocket formed by multiple residues in TM3, 6 and 7 (Kelley et al., 2001). At gpH_2R-$G_{s\alpha S}$, IMP and ARP are full agonists and 30-fold and 16-fold more potent, respectively, than HIS. At hH_2R-$G_{s\alpha S}$, IMP and ARP are only partial agonists and just 6-fold more potent than HIS (Kelley et al., 2001). Modeling and mutagenesis studies revealed that the pharmacological differences between hH_2R and gpH_2R are attributable to the non-conserved Asp-271 in TM7 of gpH_2R (Ala-271 in hH_2R) and Tyr-17 in TM1 of gpH_2R (Cys-17 in hH_2R). Furthermore, the comparison of agonist efficacies in the GTPase assay with the efficacies of agonists at stabilizing the high-affinity ternary complex of H_2R with nucleotide-free $G_{s\alpha}$ indicated that guanidines stabilize ligand-specific H_2R conformations (Kelley et al., 2001). Finally, ARP-derived compounds are H_1R antagonists with preference for gpH_1R relative to hH_1R, Asn-84 in TM2 playing a crucial role in determining species-selectivity of H_1R ligands (Seifert et al., 2003; Bruysters et al., 2005).

The aim of this study was to further probe the concept of ligand-specific H_1R- and H_2R conformations. Therefore, we analyzed the interactions of H_1R and H_2R species

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isoforms with N^G-acylated imidazolylpropylguanidines (AIPGs) which are less basic than guanidines (Ghorai, 2005). UR-PG146 (**5**) is the AIPG analog of IMP (**4**), and UR-PG136 (**7**) is the AIPG analog of ARP (**6**). In AIPGs **8**-**16**, various substituents were introduced at the imidazolylpropyl moiety, and **17** represents an imidazolylethyl analog, the shorter homolog, of UR-PG80 (**11**).

Methods

Materials. Construction of baculoviruses encoding hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, hH_1R and gpH_1R was described previously (Kelley et al., 2001; Seifert et al., 2003). Baculoviruses encoding RGS proteins 4 and 19 were a gift from Dr. E. Ross (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX). Baculovirus encoding rH_2R was a gift from Dr. C. Harteneck (Department of Pharmacology, Free University of Berlin, Germany). Guanidines **4** and **6** were synthesized as described (Durant et al., 1978; Buschauer, 1989). AIPGs **5** and **7**-**17** were prepared as described (Ghorai, 2005). Structures of synthesized compounds were confirmed by ^{1}H NMR spectroscopy and high resolution mass spectrometry. Purity of compounds was >98% as determined by high-performance liquid chromatography or capillary electrophoresis (Schuster et al., 1997). AIPGs **5** and **7**-**17** were prepared as trifluoroacetate salts to ensure water solubility. Stock solutions of compounds **1**-**17** (0.1, 1 and 10 mM) each were prepared in distilled water and stored at -20°C. Under these conditions, compounds were stable for at least 2 years (longer periods of time were not studied). Further dilutions of compounds **1**-**17** were prepared fresh daily. Sources of other materials are described elsewhere (Kelley et al., 2001; Houston et al., 2002; Seifert et al., 2003). Baculovirus infection and culture of Sf9 cells and membrane preparation were performed as described (Kelley et al., 2001). H_2R-$G_{s\alpha}$ expression levels were 5-6 pmol/mg as assessed by immunoblotting using the M1 monoclonal antibody and β_2-

adrenoceptor expressed at defined levels as standard (Kelley et al., 2001). H_1R expression levels were 4-6 pmol/mg as assessed by [^{3}H]mepyramine saturation binding (Seifert et al., 2003).

Steady-state GTPase activity assay. GTP hydrolysis in Sf9 membranes expressing H_2R-$G_{s\alpha}$ fusion proteins or H_1R isoforms plus RGS proteins was determined as described previously (Kelley et al., 2001; Seifert et al., 2003). In brief, assay tubes (100 µl) contained Sf9 membranes (10 µg of protein/tube), various ligands, 1.0 mM $MgCl_2$, 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 [γ-^{32}P]GTP (0.2-0.5 µCi/tube). Reactions were conducted for 20 min at 25^{o}C and terminated by the addition of 900 µl slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0. $^{32}P_i$ in supernatant fluids of reaction mixtures was determined by liquid scintillation counting.

AC assay. AC activity in Sf9 membranes expressing non-fused hH_2R, gpH_2R and rH_2R was determined as described previously (Houston et al., 2002). In brief, assay tubes (50 µl) contained Sf9 membranes (50-100 µg of protein/tube), various ligands, 5.0 mM $MgCl_2$, 0.4 mM EDTA, 40 µM ATP, [α-^{32}P]ATP (1.0-1.5 µCi/tube), 100 µM GTP, 100 µM cAMP and an ATP-regenerating system consisting of phosphoenolpyruvate, pyruvate kinase and myokinase in 30 mM Tris/HCl, pH 7.4. Reactions were conducted for 20 min at 37^{o}C and terminated by the addition of 20 µl of 2.2 N HCl. [α-^{32}P]ATP was separated from [^{32}P]cAMP by column chromatography as described (Liu et al., 2001), and [^{32}P]cAMP was determined by liquid scintillation counting.

Radioligand binding assays. [^{3}H]Tiotidine competition binding experiments with Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ or gpH_2R-$G_{s\alpha S}$ were performed as described previously (Kelley et al., 2001). In brief, assay tubes (250 µl) contained membranes (200-250

μg of protein/tube), 10 nM [^{3}H]tiotidine and unlabeled ligands in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). [^{3}H]Mepyramine competition binding experiments with Sf9 membranes expressing hH_1R or gpH_1R plus RGS proteins were performed as described previously (Seifert et al., 2003). In brief, assay tubes (500 μl) contained membranes (20-25 μg of protein/tube), 2 nM [^{3}H]mepyramine and unlabeled ligands in binding buffer. Bound radioligand was separated from free radioligand by filtration through GF/C filters, and filter-bound radioactivity was determined by liquid scintillation counting.

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 4.02 software (GraphPad-Prism, San Diego, CA). K_i- and K_B values were calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Statistical comparisons in Table 1 were performed with the *t*-test; statistical comparisons in Table 2 with ANOVA.

Results

Agonist potencies and efficacies of guanidines and AIPGs at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ in the GTPase assay. All AIPGs studied with the exception of **12** at hH_2R exhibited agonistic activity at H_2R isoforms (Table 1). At hH_2R-$G_{s\alpha S}$, exchange of the methylene group against a carbonyl group had little effect on the potency and efficacy of the couple **4** and **5** and moderately reduced potency but not efficacy in the couple **6** and **7**. Omission of the *p*-fluoro substituent of the phenyl group in AIPGs (**7**→**8**) had little effect on potency and efficacy. The same was true for the exchange of the pyridyl group against an imidazolyl group (**8**→**9**). Substitution of the imidazolyl ring with an additional benzyl group (**9**→**10**) substantially reduced efficacy at hH_2R. Most prominently, exchange of the pyridyl

group against a phenyl group (**8→11**) increased potency by almost tenfold, while efficacy was slightly reduced. Shortening of the linker between the carbonyl group and the phenyl rings was deleterious for agonist efficacy (**11→12**), whereas introduction of *p*-fluoro substituents at both phenyl rings (**11→15**, UR-PG55B) resulted in the most potent hH_2R agonist known so far (25-fold more potent than HIS). Changes of the fluoro substitution pattern (compare **15** with **13** and **14**) and exchange of one phenyl ring by a thiazole ring (compare **14** and **16**) reduced agonist potency. Exchange of the imidazolylpropyl group against an imidazolylethyl group (**11→17**) strongly reduced efficacy at hH_2R while only moderately reducing potency.

Potencies and efficacies of AIPGs were higher at gpH_2R-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$ (Table 1). These differences in interaction of AIPGs with hH_2R and gpH_2R resulted in correlations of efficacies (Fig. 2A) and potencies (Fig. 2B) that were shifted towards gpH_2R. A shift towards higher potencies and efficacies at gpH_2R relative to hH_2R was also observed for guanidines (Kelley et al., 2001). However, compared to the data obtained with guanidines, more AIPGs deviated from the correlation between gpH_2R and hH_2R. Most notably, AIPG **10** was more efficacious at hH_2R than at gpH_2R, and compound **17** was a similarly potent partial agonist at hH_2R and gpH_2R. AIPGs **10** and **15** were also just 2-fold more potent agonists at gpH_2R-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$.

Agonist potencies and efficacies of small H_2R agonists, guanidines and UR-PG61 at hH_2R, gpH_2R and rH_2R in the AC assay. Although the measurement of steady-state GTP hydrolysis at H_2R-$G_{s\alpha}$ fusion proteins provides a sensitive read-out for agonist potencies and efficacies independently of the effector AC, fusion proteins do not represent a physiological system (Seifert et al., 1999a). Therefore, we also determined the potencies and efficacies of representative H_2R agonists at non-fused H_2R isoforms by measuring AC activity (Table 2). At hH_2R DIM (**3**) was a 5-fold less potent agonist than HIS (**1**), whereas

AMT (**2**) was 2-fold more potent than HIS. The guanidines IMP and ARP as well as a representative AIPG (UR-PG61, **14**) were 4-6-fold more potent agonists than HIS. The efficacies of **2**-**4**, **6** and **14** at non-fused hH_2R and hH_2R-$G_{s\alpha S}$ were similar (Tables 1 and 2) (Kelley et al., 2001).

Whereas at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$, HIS is a similarly potent agonist (Table 1), HIS was 6-fold less potent at non-fused gpH_2R than at hH_2R (Table 2). A similar potency difference was observed between hH_2R and rH_2R. Therefore, agonist potencies between the three receptor systems could only be compared on the basis of relative agonist potencies, HIS being the reference for each H_2R isoform. At gpH_2R, DIM was similarly potent as HIS, while AMT was an almost 6-fold more potent agonist than HIS. ARP, IMP and UR-PG61 were up to 170-fold more potent gpH_2R agonists than HIS. In terms of efficacy, the compounds studied were all strong partial agonists, with DIM being the least efficacious agonist. In contrast, at gpH_2R-$G_{s\alpha S}$, DIM is a full agonist (Kelley et al., 2001).

At rH_2R, DIM was 2-fold less potent than HIS, whereas AMT was 6-fold more potent than HIS. IMP and ARP were up to 19-fold more potent agonists than HIS, and UR-PG61 was the most potent agonist among the compounds studied, surpassing the potency of HIS by 67-fold. At rH_2R, ARP was almost a full agonist; **2**-**4** were strong partial agonists and compound **14** exhibited only moderate efficacy.

Ternary complex formation at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. Agonists stabilize a high-affinity ternary complex with GPCR and the guanine nucleotide-free G-protein (De Lean et al., 1980; Seifert et al., 1998, 1999b). In many systems, stable GTP analogs such as GTPγS disrupt the ternary complex and, thereby, reduce the agonist-affinity of GPCR (De Lean et al., 1980; Seifert et al., 1998, 1999b). Interestingly, various guanidines differentially stabilize the ternary complex at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$, pointing to the existence of

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agonist-specific H_2R conformations (Kelley et al., 2001). Fig. 3 shows the agonist competition curves of UR-PG146 (**5**) and UR-PG136 (**7**) on [^{3}H]tiotidine (antagonist) binding to hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ in the absence and presence of GTPγS. Table 3 provides a summary of the binding properties of **5** and **7** as well as the corresponding guanidines IMP (**4**) and ARP (**6**). UR-PG146 did not measurably stabilize the ternary complex in hH_2R-$G_{s\alpha S}$ as is indicated by the missing rightward-shift of the agonist competition curve in the presence of GTPγS. In contrast, GTPγS shifted the IMP competition curve at hH_2R-$G_{s\alpha S}$ 4-fold to the right. Compared to hH_2R-$G_{s\alpha S}$, UR-PG146 was much more efficient at stabilizing the ternary complex at gpH_2R-$G_{s\alpha S}$ as is indicated by the high fraction of high-affinity binding sites in the absence of GTPγS and the strong rightward-shift of the agonist competition curve by GTPγS. UR-PG146 stabilized the ternary complex at gpH_2R-$G_{s\alpha S}$ more efficiently than IMP.

In contrast to UR-PG146, UR-PG136 efficiently stabilized the ternary complex at hH_2R-$G_{s\alpha S}$ as is indicated by the strong rightward-shift of the agonist competition curve by GTPγS. ARP also stabilized the ternary complex at hH_2R-$G_{s\alpha S}$, but unlike with UR-PG136, distinct high-affinity binding sites were discriminated with ARP. UR-PG136 stabilized the ternary complex in gpH_2R-$G_{s\alpha S}$ less efficiently than in hH_2R-$G_{s\alpha S}$ as is evident from the smaller shift of the agonist competition curve by GTPγS. At gpH_2R-$G_{s\alpha S}$ ARP was an efficient stabilizer of the ternary complex, but this ternary complex formation was insensitive to guanine nucleotides as is seen from the preservation of distinct high-affinity binding sites in the presence of GTPγS.

Interaction of guanidines and AIPGs with the H_1R. Since guanidines are H_1R antagonists with up to 10-fold selectivity for the gpH_1R relative to the hH_1R (Seifert et al., 2003), we also examined the interactions of AIPGs with H_1R. Compared to the agonist HIS,

the antagonist ARP exhibited 6-fold higher affinity to hH_1R in [^{3}H]mepyramine competition binding experiments, and the affinity of ARP to gpH_1R was 150-fold higher (Table 4). IMP exhibited selectivity for the gpH_1R relative to the hH_1R as well. Strikingly, the exchange of a methylene group against a carbonyl group (**4**→**5** and **6**→**7**) reduced the affinity of AIPGs for H_1Rs up to ~300-fold. In general, AIPGs exhibited higher affinity for gpH_1R than for hH_1R, but the gpH_1R-selectivity for AIPGs was less pronounced than for guanidines (**4**→**5** and **6**→**7**). Additionally, in case of UR-PG131A (**9**) and UR-PG55B (**15**), affinity for both H_1R isoforms was similar.

To answer the question whether AIPGs are H_1R agonists or antagonists, we examined the effects of the compounds on GTPase activity (Table 5). AIPGs exhibited weak to moderate partial agonistic activity at hH_1R, with UR-PG126 (**16**) being the most efficacious compound. AIPGs were 10-70-fold less potent than HIS at hH_1R. At gpH_1R, AIPGs were considerably less efficacious partial agonists than at hH_1R, rendering calculation of agonist potencies impossible. For those compounds, antagonist potencies were calculated. The GTPase antagonist studies corroborated the notion that AIPGs exhibit only low affinity for hH_1R and gpH_1R with K_B values in the 2-15 μM range. Noteworthy, in the functional antagonist assay, AIPG **11** exhibited 2-fold higher affinity for hH_1R than for gpH_1R.

Discussion

Previous studies with HL-60 promyelocytes and H_2R-$G_{s\alpha}$ fusion proteins provided the first evidence for the notion that H_2R agonists stabilize distinct ligand-specific H_2R conformations, i.e. multiple active H_2R states (Gespach et al., 1982; Seifert et al., 1992; Kelley et al., 2001; Wenzel-Seifert et al., 2001). A multiple-state model is a fundamental concept since it implies more versatile manipulation of GPCR-mediated signaling than within

a two-state model assuming a single inactive (R) and a single active (R*) state (Seifert and Wenzel-Seifert, 2002; Kenakin 2003). An increasing number of reports indicate that ligand-specific active states are a general property of GPCRs encompassing adrenoceptors, dopamine receptors, serotonin receptors and cannabinoid receptors (Seifert et al., 1999b, 2001; Villazon et al., 2003; Gay et al., 2004; Clarke 2005; Mukhopadhyay and Howlett, 2005). With respect to the H_1R and H_2R, ARP-derived guanidines are particularly useful conformational probes since these ligands discriminate between species isoforms of those GPCRs (Kelley et al., 2001; Wenzel-Seifert et al., 2001; Seifert et al., 2003). These data prompted us to examine a series of ARP-derived compounds in which the N^G-alkyl substituent was replaced against an N^G-alkanoyl group (compare **4** and **6** versus **5** and **7**-**17**) (Fig. 1). The resulting AIPGs are less basic than the corresponding guanidines. According to a two-state model, a change in basicity would be expected to result in quantitative changes in interactions of compounds with GPCRs, whereas within the framework of a multiple-state model, qualitative changes would be expected to occur.

The AIPG UR-PG55B (**15**) is the most potent hH_2R agonist known so far, surpassing the potency of ARP, the prototypical guanidine, by almost 4-fold (Table 1). However, at gpH_2R-$G_{s\alpha}$, UR-PG80 (**11**) rather than **15** is the most potent gpH_2R agonist. Whereas ARP-derived guanidines exhibit similar affinity for the couple hH_1R/hH_2R and gpH_1R/gpH_2R (Kelley et al., 2001; Seifert et al., 2003), AIPGs exhibit up to 1,000-fold selectivity for H_2R relative to H_1R (Tables 1, 4 and 5). These differences between AIPGs and guanidines were the first indication for distinct interactions of AIPGs with H_1R and H_2R.

Ionic interaction of the amino group of HIS and the guanidino group of IMP, ARP and related compounds with Asp-98 in TM3 is important for high-affinity ligand/H_2R interaction (Gantz et al., 1992; Kelley et al., 2001). Regardless of the reduced pK_a values (in the range of 7-8 for AIPGs compared to about 12.5 for guanidines), the compounds are

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sufficiently basic to form an ionic interaction or charge-assisted hydrogen bond of the N^G-acylguanidino group with Asp-98 at physiological pH. The geometry of both series of compounds, N^G-alkylguanidines and N^G-acylguanidines, is sufficiently similar to assume comparable binding modes to H_2R (Fig. 1). Surprisingly, certain AIPGs even surpass guanidines in terms of agonistic potency at H_2R isoforms from various species (Tables 1 and 2). With respect to AIPG substitution, the most striking result is that the exchange of the pyridyl group against a second phenyl group (**8**→**11**) increased agonist potency up to ten-fold (Fig. 1 and Table 1). For hH_2R, the increase in affinity of AIPGs by the diphenyl substitution was expected since Ala-271 in TM7 facilitates hydrophobic interactions (Kelley et al., 2001). However, in gpH_2R, the pyridyl group of ARP participates in ion dipole interactions with Asp-271 which cannot take place with a phenyl ring (Kelley et al., 2001). These data are explained by a model in which AIPG **11** adopts a different orientation in gpH_2R than guanidines, allowing it to interact with hydrophobic amino acids present in TM3, 6 and 7.

While, in general, AIPGs are more potent and efficacious at gpH_2R than at hH_2R (Fig. 2), we observed some exceptions from this rule. Notably, hH_2R tolerates introduction of an additional benzyl group at the imidazolyl group better in terms of efficacy than gpH_2R (**9**→**10**) (Fig. 1 and Table 1). In addition, hH_2R tolerates an imidazolylethyl group better concerning agonist potency than gpH_2R (**11**→**17**). Moreover, AIPGs **10** and **15** are similarly potent agonists at hH_2R and gpH_2R. The potency-enhancing effect of the second phenyl ring in both hH_2R and gpH_2R (**8**→**11**) and the distinct structure-activity relationships of AIPGs at hH_2R and gpH_2R (**9**→**10** and **11**→**17, 10**, **15**) in the GTPase assay prompted us to examine ternary complex formation since this parameter is sensitive at unmasking ligand-specific GPCR conformations (Seifert et al., 2001; Kelley et al., 2001). Indeed, although the couples IMP (**4**)/UR-PG-146 (**5**) and ARP (**6**)/UR-PG-136 (**7**) resemble each other with respect to efficacy in the GTPase assay (Table 1), the compounds differ substantially from each other

regarding ternary complex formation (Fig. 3 and Table 3). At gpH_2R-$G_{s\alpha S}$, UR-PG146 was particularly efficient at ternary complex formation. Efficient ternary complex formation that is not accompanied by a correspondingly high efficacy in terms of steady-state GTP turnover is indicative for formation of non-signaling (frozen) ternary complexes (Seifert et al., 2001; Kenakin, 2003). The existence of ligand-specific H_2R conformations stabilized by AIPGs is further supported by the fact that at hH_2R, the $K_{lGTP\gamma S}$ values of UR-PG146 (**5**) and UR-PG-136 (**7**) are similar to agonist potencies in the GTPase assay, suggesting that the low agonist-affinity state of hH_2R bound to AIPG promotes efficient guanine nucleotide exchange. Efficient coupling of the low agonist-affinity state of GPCR to G-proteins in terms of guanine nucleotide exchange was also shown for the human formyl peptide receptor (Wenzel-Seifert et al., 1999). In contrast, at gpH_2R, the K_h value of UR-PG146 (**5**) resembles the EC_{50} value in the GTPase assay, suggesting that the high-affinity state of gpH_2R bound to **5** mediates guanine nucleotide exchange.

We performed structure-activity relationship studies on guanidines and AIPGs with H_2R-$G_{s\alpha}$ fusion proteins measuring the outcome of ligand-receptor interactions at a proximal point of the signal transduction cascade, namely the steady-state GTPase activity (Kelley et al., 2001) (Table 1). This approach ensured comparison of H_2R species isoforms under identical experimental conditions with the endogenous agonist HIS (**1**) being similarly potent at both GPCRs. Complementary studies with non-fused hH_2R, gpH_2R and rH_2R in the AC assay corroborated the notion that guanidines and AIPGs are potent H_2R agonists (Table 2). Surprisingly however, in contrast to fusion proteins, up to 6-fold differences in HIS potency between non-fused H_2R isoforms were observed (Tables 1 and 2). In addition, at non-fused H_2Rs, we observed species-differences in potency and efficacy among the small synthetic agonists AMT (**2**) and DIM (**3**) (Table 2) that were not apparent in the corresponding fusion proteins (Kelley et al., 2001) (Table 2). Moreover, like hH_2R, rH_2R contains Ala-271 in TM7

(Ruat et al., 1991). Accordingly, we expected similar potencies of guanidines and AIPGs at hH_2R and rH_2R in the AC assay, but this was not the case using the relative potency of HIS (**1**) as reference (Table 2). Most prominently, the relative potency of **14** at rH_2R was more than 10-fold higher than at hH_2R. Furthermore, the system with non-fused H_2Rs did not reveal the species-differences in efficacy of guanidines and AIPGs seen in the fusion proteins (Kelley et al., 2001) (Tables 1 and 2). Instead, the small agonist DIM (**3**) exhibited lower efficacy at gpH_2R than at hH_2R. The different agonist profiles of H_2Rs in the GTPase and AC assay fit to the concept of ligand-specific GPCR conformations which differ from each other in their ability to promote guanine nucleotide exchange at $G_{s\alpha}$ relative to AC activation. Dissociations in ligand efficacies at promoting nucleotide exchange relative to AC activation were previously reported for the β_2-adrenoceptor fused to $G_{s\alpha}$ (Seifert et al., 1999b).

Whereas for the H_2R, the exchange of a methylene group against a carbonyl group had little impact on ligand potency (Fig. 1 and Table 1), this exchange substantially decreased the affinity of compounds for H_1R (compare couple **6/7** in Tables 4 and 5 and couple **4/5** in Table 4) and, thereby, increased H_2R-selectivity. Unexpectedly, we also observed a change in quality of the effects of AIPGs at H_1R. Specifically, most AIPGs are partial hH_1R agonists (Table 5) whereas guanidines are H_1R antagonists (Seifert et al., 2003). The effects of AIPGs and guanidines were studied side by side and in membranes expressing hH_1R and gpH_1R at similar expression levels, ruling out differences in GPCR expression level or GPCR/G-protein stoichiometry accounting for the differences between the two classes of compounds. These data indicate that guanidines and AIPGs also stabilize distinct H_1R conformations. It is particularly noteworthy that AIPGs exhibit higher efficacies at hH_1R than at gpH_1R (Table 5). Moreover, some AIPGs exhibit similar (**9**, **15**) or higher affinity (**11**) for hH_1R relative to gpH_1R (Tables 4 and 5). All bulky agonists studied so far exhibited preference for gpH_1R relative to hH_1R in terms of affinity and/or efficacy (Seifert et al., 2003).

In conclusion, AIPGs stabilize different active conformations in hH_2R, gpH_2R and rH_2R than guanidines. Moreover, AIPGs are more efficient at stabilizing a partially active state in hH_1R than in gpH_1R. Our data corroborate the concept that a multiple-state model is more appropriate to describe ligand/GPCR interactions than a two-state model.

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Footnotes

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Legends for Figures

Fig. 1. Structures of H_2R agonists. Compounds **1-3** represent small H_2R agonists; compounds **4-17** represent bulky H_2R agonists. **4** and **6** are guanidines, **5** and **7-17** are AIPGs. Note that **4** and **5** as well as **6** and **7** represent guanidine/AIPG couples. **17** Represents the imidazolylethyl analog of **11**.

Fig. 2. Correlation between efficacies and potencies of AIPGs at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. Agonist efficacies were taken from Table 1, and pEC_{50} values were derived from the EC_{50} values shown in Table 1. Solid lines represent the actual correlations obtained. Dashed lines represent the 95% confidence intervals of the correlations. The straight dotted lines represent the correlations that would have been obtained if efficacies and pEC_{50} values, respectively, had been identical in the two systems compared with each other. The theoretical curves have a slope of 1.00. **A**, correlation of efficacies of AIPGs at hH_2R-$G_{s\alpha S}$ *versus* gpH_2R-$G_{s\alpha S}$. Slope, 0.72 ± 0.17; $r^2 = 0.64$; $p = 0.0019$ (significant). **B**, correlation of potencies of AIPGs at hH_2R-$G_{s\alpha S}$ *versus* gpH_2R-$G_{s\alpha S}$. Slope, 1.00 ± 0.24; $r^2 = 0.65$; p = 0.0027 (significant).

Fig. 3. Competition of [^{3}H]tiotidine binding by AIPGs in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. [^{3}H]Tiotidine binding was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes (200-250 μg of protein per tube) expressing fusion proteins, 10 nM [^{3}H]tiotidine and AIPGs at the concentrations indicated on the abscissa. Reaction mixtures additionally contained distilled water (control) or GTPγS (10 μM). **A** and **B**, analysis of UR-PG136 (**7**); **C** and **D**, analysis of UR-PG146 (**5**). Data were analyzed for best fit to monophasic and biphasic competition curves (F test). Data points shown are the means ± S.D. of 5-7 experiments performed in duplicate.

Tables

Table 1. Agonist potencies and efficacies of HIS, guanidines and AIPGs at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ in the GTPase assay

			hH_2R-$G_{s\alpha S}$			gpH_2R-$G_{s\alpha S}$		EC_{50} hH_2R-$G_{s\alpha S}$/ EC_{50} gpH_2R-$G_{s\alpha S}$
Cpd.		efficacy	EC_{50} (nM)	rel. pot.	efficacy	EC_{50} (nM)	rel. pot.	
1	**HIS**	**1.00**	**1,200 ± 300**	**100**	**1.00**	**1,200 ± 200**	**100**	**1.00**
4	**IMP**	**0.82 ± 0.04**	**210 ± 20***	**570**	**0.99 ± 0.09**	**42 ± 10**	**2,900**	**5.00**
5	**UR-PG146**	**0.79 ± 0.04***	**270 ± 38***	**440**	**0.93 ± 0.01**	**60 ± 1**	**2,000**	**4.44**
6	**ARP**	**0.80 ± 0.05***	**180 ± 50***	**670**	**1.00 ± 0.06**	**65 ± 8**	**1,800**	**2.77**
7	**UR-PG136**	**0.73 ± 0.03***	**420 ± 90***	**290**	**0.93 ± 0.04**	**45 ± 4**	**2,700**	**9.21**
8	**UR-PG122A**	**0.86 ± 0.01***	**550 ± 33***	**220**	**1.03 ± 0.07**	**60 ± 14**	**2,000**	**9.17**
9	**UR-PG131A**	**0.88 ± 0.10**	**650 ± 92***	**180**	**0.90 ± 0.01**	**260 ± 10**	**460**	**2.51**
10	**UR-PG137**	**0.38 ± 0.01***	**780 ± 52***	**160**	**0.28 ± 0.01**	**360 ± 38**	**340**	**2.18**
11	**UR-PG80**	**0.69 ± 0.09**	**78 ± 42***	**1,500**	**0.93 ± 0.32**	**6 ± 1**	**19,000**	**12.1**
12	**UR-PG123**	**0.08 ± 0.04***	**n. a.**	**n.a.**	**0.60 ± 0.11**	**400 ± 86**	**300**	**n. a.**
13	**UR-PG59**	**0.67 ± 0.07***	**110 ± 34***	**1,100**	**1.00 ± 0.11**	**20 ± 15**	**5,900**	**5.56**
14	**UR-PG61**	**0.72 ± 0.09***	**61 ± 15***	**2,000**	**1.02 ± 0.11**	**7 ± 1**	**16,000**	**8.23**
15	**UR-PG55B**	**0.61 ± 0.02***	**48 ± 16**	**2,500**	**0.81 ± 0.13**	**25 ± 17**	**4,800**	**1.88**
16	**UR-PG126**	**0.76 ± 0.01***	**300 ± 4***	**400**	**1.01 ± 0.09**	**75 ± 5**	**1,600**	**3.96**
17	**UR-PG153**	**0.17 ± 0.01***	**190 ± 9**	**630**	**0.54 ± 0.02**	**180 ± 27**	**700**	**1.06**

Steady-state GTPase activity in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ was determined as described in *Methods*. Reaction mixtures contained ligands 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 basal GTPase activities ranged between ~1 - 2 pmol/mg/min, and the maximum stimulatory effect of histamine (100 μM) amounted to 250 - 350% above basal. 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 hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. 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 EC_{50} values of H_2R agonists for hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$. n. a., not applicable; Cpd., compound.

Table 2. Agonist potencies and efficacies of small H_2R agonists, guanidines and UR-PG61 at hH_2R, gpH_2R and rH_2R in the AC assay

			hH_2R			gpH_2R			rH_2R	
Cpd.		**efficacy**	**EC_{50} (nM)**	**rel. pot.**	**efficacy**	**EC_{50} (nM)**	**rel. pot.**	**efficacy**	**EC_{50} (nM)**	**rel. pot.**
1	HIS	1.00	$390 \pm 7^{*,+}$	100	1,00	2,200 ± 720	100	1.00	2,200 ± 250	100
2	AMT	$1.01 \pm 0.03^{+}$	$190 \pm 29^{*,+}$	210	0.92 ± 0.06	500 ± 42	570	0.86 ± 0.02	360 ± 79	640
3	DIM	$0.87 \pm 0.02^{*}$	$1,700 \pm 150^{+}$	23	0.70 ± 0.02	2,200 ± 310	130	$0.85 \pm 0.01^{\#}$	$5,100 \pm 1,100^{\#}$	45
4	IMP	0.88 ± 0.01	$95 \pm 3^{*}$	410	0.87 ± 0.01	28 ± 14	14,000	0.82 ± 0.07	$120 \pm 13^{\#}$	1,900
6	ARP	0.79 ± 0.05	$63 \pm 1^{*,+}$	620	0.86 ± 0.07	17 ± 1	17,000	0.97 ± 0.15	$160 \pm 32^{\#}$	1,400
14	UR-PG61	$0.80 \pm 0.03^{+}$	$68 \pm 6^{*}$	580	0.84 ± 0.03	29 ± 5	10,000	$0.46 \pm 0.04^{\#}$	61 ± 42	6,700

AC activity in Sf9 membranes expressing non-fused hH_2R, gpH_2R or rH_2R was determined as described in *Methods*. Reaction mixtures contained 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 and maximum HIS-stimulated AC activities were as follows. hH_2R; 1.5 and 5.0 pmol/mg/min, respectively; gpH_2R, 1.5 and 5.0 pmol/mg/min, respectively; rH_2R, 0.7 and 3.0 pmol/mg/min, respectively. 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 4-5 experiments performed in duplicates each. * $p < 0.05$ for comparison of hH_2R and gpH_2R; $^{+}$ $p < 0.05$ for comparison of hH_2R and rH_2R; $^{\#}$ $p < 0.05$ for comparison of gpH_2R and rH_2R. The relative potency (rel. pot.) of histamine was set 100, and the potencies of other agonists were referred to this value.

Table 3. Agonist binding properties of guanidines and AIPGs at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$

Cpd.		K_h (nM)	K_l (nM)	$\%R_h$	$K_{hGTP\gamma S}$ (nM)	$K_{lGTP\gamma S}$ (nM)	$\%R_{hGTP\gamma S}$ (nM)
	hH_2R-$G_{s\alpha S}$						
4	IMP	-	62 (44-77)	-	-	240 (160-340)	-
5	UR-PG146	-	1,100 (600-1,900)	-	-	580 (230-1,500)	-
6	ARP	10 (7.0-17)	450 (230-1,200)	61.0 (49.5-77.8)	-	320 (210-390)	-
7	UR-PG136	-	100 (37-280)	-	-	910 (450-1,800)	-
	gpH_2R-$G_{s\alpha S}$						
4	IMP	-	24 (20-34)	-	13.5 (8.5-32.0)	250 (120-490)	31.5 (12.8-44.5)
5	UR-PG146	18 (4.8-65)	2,700 (540-13,000)	55.0 (38.5 -71.4)	-	570 (390-850)	-
6	ARP	14 (6.0-29)	160 (15-380)	75.5 (51.6-87.5)	9.5 (4.6-27.0)	155 (45-390)	52.5 (22.7-70.5)
7	UR-PG136	-	43 (30-64)	-	-	150 (110-210)	-

Agonist competition binding was determined as described under *Methods*. Data shown in Fig. 3 were analyzed by non-linear regression for best fit to monophasic or biphasic competition curves. Data shown are the means of 5-7 experiments performed in duplicate. Numbers in parentheses represent the 95% confidence intervals. K_h and K_l designate the dissociation constants for the high- and low-affinity state of H_2Rs, respectively. $\%R_h$ indicates the percentage of high-affinity binding sites. The corresponding values in the presence of GTPγS (10 μM) are referred to as $K_{hGTP\gamma S}$, $K_{lGTP\gamma S}$ and $\%R_{hGTP\gamma S}$, respectively. If data were best fit to monophasic curves, data are listed under K_l and $K_{lGTP\gamma S}$, respectively.

Table 4. Affinities of HIS, guanidines and AIPGs at hH_1R and gpH_1R in the [^{3}H]mepyramine competition binding assay

		hH_1R		gpH_1R		
Cpd.		**K_i (µM)**	**rel. aff.**	**K_i (µM)**	**rel. aff.**	**aff. ratio gp/h**
1	HIS	2.0 ± 0.19	100	4.6 ± 0.24	100	0.43
4	IMP	6.0 ± 1.3	33	0.92 ± 0.14	500	6.53
5	UR-PG146	41 ± 0.91	4.9	11 ± 5.6	42	3.67
6	ARP	0.34 ± 0.08	590	0.03 ± 0.01	15,000	11.3
7	UR-PG136	29 ± 8.5	6.9	9.6 ± 3.3	48	3.01
9	UR-PG131A	22 ± 4.6	9.0	20 ± 7.6	23	1.09
15	UR-PG55B	6.7 ± 0.62	30	4.9 ± 1.3	94	1.37
17	UR-PG153	20 ± 0.09	10	6.4 ± 0.12	72	3.17

[^{3}H]Mepyramine competition binding in Sf9 membranes expressing hH_1R or gpH_1R with RGS4 or RGS19 was determined as described in *Methods*. Reaction mixtures contained Sf9 membranes (20-25 µg of protein), 2 nM [^{3}H]mepyramine and unlabeled ligands at concentrations of 10 nM - 1 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 duplicate. The relative affinity of HIS (rel. aff.) was set 100, and the affinities of other ligands were referred to this value. We also calculated the ratio of the K_B values for hH_1R and gpH_1R (aff. ratio gp/h).

Table 5. Agonist potencies and efficacies of HIS and AIPGs and antagonist potencies of AIPGs and ARP at hH_1R and gpH_1R in the GTPase assay

		hH_1R		gpH_1R	
Cpd.		**Efficacy**	**EC_{50} or K_B (nM)**	**efficacy**	**EC_{50} or K_B (nM)**
1	HIS	1.00	190 ± 8.6	1.00	210 ± 5.3
4	IMP	0.00 ± 0.01	n. a.	0.01 ± 0.01	n. d.
5	UR-PG146	0.37 ± 0.07	12,000 ± 450	0.08 ± 0.02	2,000 ± 630 (K_B)
6	ARP	0.00 ± 0.01	320 ± 80 (K_B)	0.01 ± 0.01	48 ± 15 (K_B)
7	UR-PG136	0.28 ± 0.03	9,600 ± 3,600	0.04 ± 0.02	10,000 ± 3,100 (K_B)
9	UR-PG131A	0.32 ± 0.03	10,000 ± 1,600	0.03 ± 0.01	13,400 ± 5,800 (K_B)
11	UR-PG80	0.19 ± 0.02	3,500 ± 900 (K_B)	0.03 ± 0.01	7,100 ± 570 (K_B)
14	UR-PG61	0.19 ± 0.04	6,100 ± 40 (K_B)	0.07 ± 0.04	2,700 ± 540 (K_B)
15	UR-PG55B	0.36 ± 0.12	2,000 ± 1,100	0.10 ± 0.03	n. d.
16	UR-PG126	0.42 ± 0.05	5,500 ± 1,600	0.12 ± 0.02	n. d.
17	UR-PG153	0.35 ± 0.02	2,300 ± 490	0.10 ± 0.02	2,000 ± 540 (K_B)

Steady-state GTPase activity in Sf9 membranes expressing hH_1R and gpH_1R in the presence of the RGS proteins 4 or 19 was determined as described in *Methods*. Reaction mixtures contained 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 (100 μM) amounted to 125 - 175% above basal. 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. The relative potency (rel. pot.) of histamine at hH_1R was set 100, and the potencies of other agonists were referred to this value. With several AIPGs, particularly with gpH_1R, the stimulatory effects were too small to calculate agonist potencies. In those cases, efficacies with agonist at a fixed concentration (100 μM) and K_B values (determined in the presence of 1 μM HIS) were calculated. n. d., not determined; n. a., not applicable.

Fig. 1

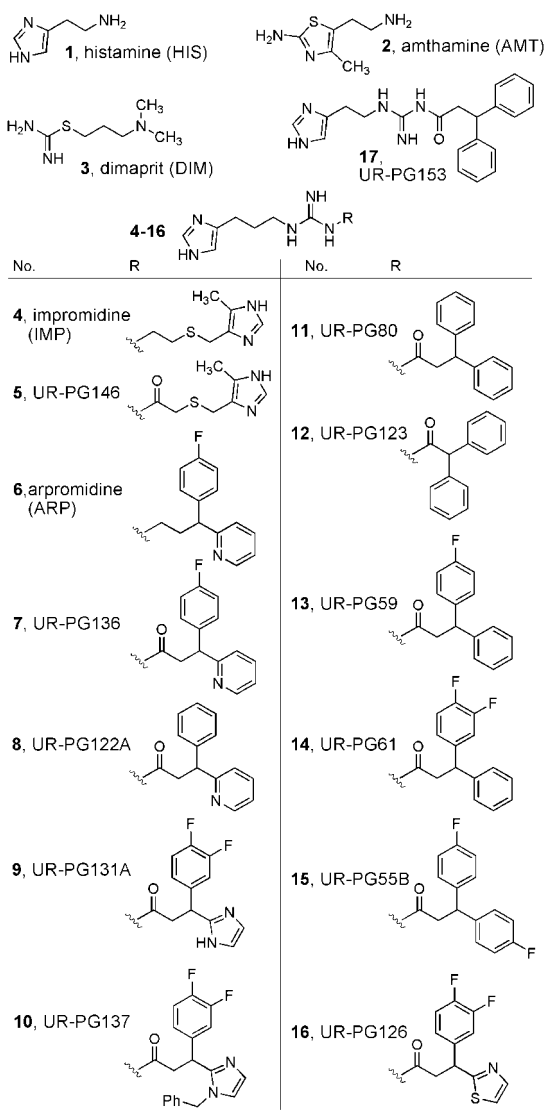

Fig. 2

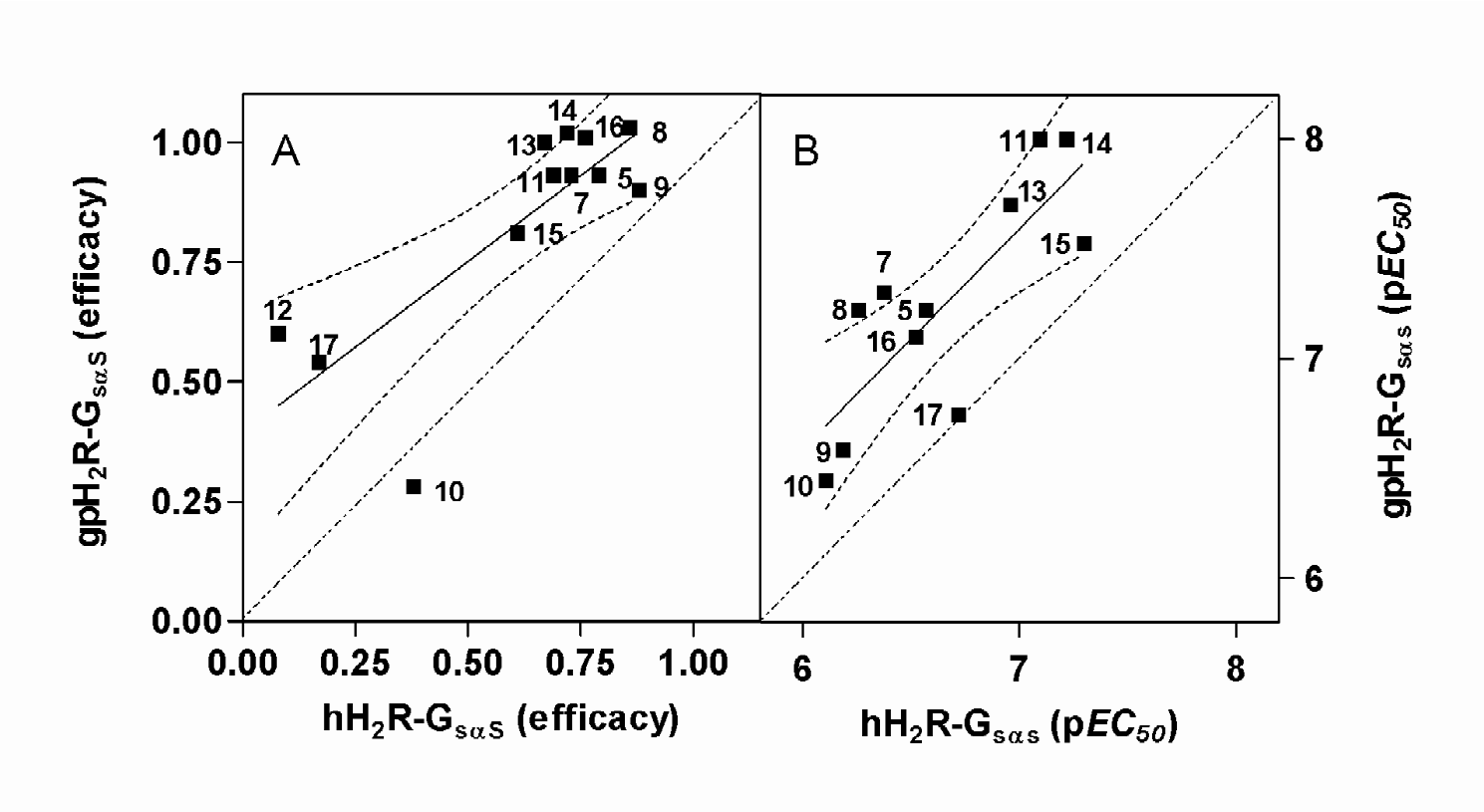

Fig. 3