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N^1-(3-Cyclohexylbutanoyl)-N^2-[3-(1*H*-imidazol-4-yl)propyl]guanidine, a potent partial agonist for the human histamine H_1- and H_2-receptor

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Running title: H_1- and H_2-receptor activation by imidazolylpropylguanidines

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Abbreviations used: AIPG, N^G-acylated imidazolylpropylguanidine; 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 $G_{s\alpha}$; 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; RGS protein, regulator of G-protein signaling; TM, transmembrane domain.

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Abstract

Both the histamine H_1-receptor (H_1R) and H_2-receptor (H_2R) exhibit pronounced species-selectivity in their pharmacological properties, i.e. bulky agonists possess higher potencies and efficacies at guinea pig (gp) than at the corresponding human (h) receptor isoforms. In this study, we examined the effects of N^G-acylated imidazolylpropylguanidines substituted with a single phenyl- or cyclohexyl substituent on H_1R and H_2R species isoforms expressed in Sf9 insect cells. N^1-(3-Cyclohexylbutanoyl)-N^2-[3-(1*H*-imidazol-4-yl)propyl]guanidine (UR-AK57) turned out to be the most potent hH_2R agonist identified so far (EC_{50} of 23 nM in the GTPase assay at the hH_2R-$G_{s\alpha}$ fusion protein expressed in Sf9 insect cells). UR-AK57 was almost a full hH_2R agonist and only slightly less potent and efficacious than at gpH_2R-$G_{s\alpha}$. Several N^G-acylated imidazolylpropylguanidines showed similar potency at hH_2R and gpH_2R. Most unexpectedly, UR-AK57 exhibited moderately strong partial hH_1R agonism with a potency similar to that of histamine, whereas at gpH_1R, UR-AK57 was only a very weak partial agonist. Structure/activity relationship studies revealed that both the alkanoyl chain connecting the aromatic or alicyclic substituent with the guanidine moiety and the nature of the carbocycle (cyclohexyl *versus* phenyl ring) critically determine the pharmacological properties of this class of compounds. Collectively, our data show that gpH_1R and gpH_2R do not necessarily exhibit preference for bulky agonists compared to hH_1R and hH_2R, respectively, and that UR-AK57 is a promising starting point for the development of both potent and efficacious hH_1R- and hH_2R agonists.

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Introduction

Histamine (HIS) (**1**) (Fig. 1) is a neurotransmitter and autacoid and acts through H_1-, H_2-, H_3- and H_4-receptors (Hill et al., 1997; Hough, 2001; Bakker et al., 2002). The H_1R couples to G_q-proteins to mediate phospholipase C activation and plays a role in the regulation of alertness and as mediator of type 1 allergic reactions (Hill et al., 1997; Bakker et al., 2002). The H_2R couples to G_s-proteins to mediate adenylyl cyclase activation and regulates H^+ secretion in gastric parietal cells, cardiac contractility and various myeloid cell functions (Klinker et al., 1996; Hill et al., 1997; Bakker et al., 2002).

It has been difficult to establish relevant native test systems for the analysis of the human H_1R (hH_1R) and human H_2R (hH_2R) since there are unexplained pharmacological differences in the properties of hH_1R and hH_2R in native cells relative to standard guinea pig test organs (Burde et al., 1990; Seifert et al., 1994; Klinker et al., 1996). In order to facilitate the comparison of histamine receptors under identical experimental conditions, we established expression systems for the H_1R and H_2R in Sf9 insect cells (Kelley et al., 2001; Houston et al., 2002). Sf9 cells express the H_1R and H_2R at high levels and can be cultured in large quantities. GPCR/G-protein coupling in Sf9 membranes is monitored with high sensitivity using the steady-state GTPase assay. This assay assesses GPCR/G-protein coupling at a proximal point of the signaling cascade, avoiding potential bias introduced by assessing more downstream events such as effector activation or changes in gene expression. For the H_1R, coupling of the GPCR to insect cell G_q-proteins is determined and the GTPase signal is amplified by RGS proteins (Houston et al., 2002; Seifert et al., 2003). For the H_2R, fusion proteins of GPCR and mammalian $G_{s\alpha}$ proteins ensure defined 1 : 1 stoichiometry of the coupling partners and their efficient interaction (Seifert et al., 1999; Kelley et al., 2001). By measuring GTP hydrolysis, potencies and efficacies of H_2R agonists are assessed in an

expression level-independent manner (Seifert et al., 1999; Kelley et al., 2001; Wenzel-Seifert et al., 2001).

Both H_1R- and H_2R agonists are important pharmacological tools for studying the role of the H_1R and H_2R, respectively, in (patho)physiological processes (Bakker et al., 2002; Dove et al., 2004; Pertz et al., 2004). H_1R agonists are divided into three classes; *1)* small agonists derived from HIS such as 2-methylhistamine and 2-(2-thiazolyl)ethanamine, *2)* HIS derivatives with a bulkier aromatic substituent at position 2 of the imidazole ring such as 2-(3-bromophenyl)histamine and *3)* the histaprodifens (Bakker et al., 2002; Pertz et al., 2004). Unfortunately, bulky H_1R agonists exhibit considerably lower potency and efficacy at the hH_1R than at the guinea pig H_1R (gpH_1R), limiting their usefulness as tools for studying the hH_1R (Seifert et al., 2003). The molecular basis for the differences in pharmacological properties between hH_1R and gpH_1R has recently been elucidated (Bruysters et al., 2005). A further complication is that at concentrations in the 10 μM - 1 mM range, 2-phenylhistamines may activate G-proteins directly, i.e. in a receptor-independent manner (Seifert et al., 1994; Hagelüken et al., 1995; Klinker et al., 1996).

H_2R agonists are divided into two classes; *1)* small agonists derived from HIS (**1**) such as dimaprit and amthamine, *2)* long-chained and more bulky molecules such as the guanidines arpromidine and impromidine (Bakker et al., 2002; Dove et al., 2004) and the recently introduced N^G-acylated imidazolylpropylguanidines (AIPGs) which are less basic than guanidines (Xie et al., 2006). Similar to the situation with H_1R species isoforms, bulky H_2R agonists are considerably less potent and efficacious at hH_2R than at gpH_2R, reducing their value as probes to examine hH_2R (Kelley et al., 2001; Wenzel-Seifert et al., 2001; Xie et al., 2006). The pharmacological differences between hH_2R and gpH_2R are attributable to two amino acid differences in transmembrane domains 1 and 7 (Kelley et al., 2001; Dove et al., 2004).

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The AIPGs characterized so far possess two ring systems, i.e. either two phenyl rings, a phenyl- and a pyridyl ring, a phenyl- and an imidazolyl ring or a phenyl ring and a thiazole ring (Xie et al., 2006). In our present study, we examined AIPGs substituted with a single phenyl ring (**2-8**) or a single cyclohexyl ring (**9-14**), possessing various linker lengths and alkanoyl chain branching between the acylguanidine moiety and the ring system (Fig. 1). Within this series of AIPGs, UR-AK57 (**14**) is the most potent hH_2R agonist identified so far, and surprisingly, this compound is also a potent partial hH_1R agonist.

Materials and 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). AIPGs **2-14** were prepared according to the procedure described by 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). Stock solutions of compounds **2-14** (10 mM) were prepared in dimethyl sulfoxide 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 **2-14** were prepared in distilled water. 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

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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.

[^{3}H]Mepyramine binding assay. [^{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 (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). 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.

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Results

Agonist potencies and efficacies of AIPGs 2-14 at hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ in the GTPase assay. In membranes expressing hH_2R-$G_{s\alpha S}$ (Fig. 2A) and gpH_2R-$G_{s\alpha S}$ (Fig. 2B), HIS activated GTP hydrolysis with an EC_{50} value of ~1 μM. UR-AK57 was a 50-fold more potent agonist (EC_{50}, 23 nM) at hH_2R than HIS. At gpH_2R, UR-AK57 activated GTP hydrolysis 130-fold more potently (EC_{50}, 9 nM) than HIS. At hH_2R, UR-AK57 was almost a full agonist (E_{max}, 0.87), and at gpH_2R, UR-AK57 was a full agonist (E_{max}, 1.11). UR-AK57 constitutes the most potent hH_2R agonist identified so far and surpasses the previous leader, UR-PG55B, which is substituted with two p-fluorophenyl groups, in terms of potency by two-fold (Xie et al., 2006). Moreover, UR-AK57 clearly surpasses UR-PG55B (E_{max}, 0.61) in terms of efficacy (Xie et al., 2006). Shortening of the connecting chain (**13** *versus* **14**) reduced potency and efficacy. Exchange of the 3-cyclohexylbutanoyl group in compound **14** against a 4-cyclohexylbutanoyl residue (**12**) had little effect on efficacy and potency; the same was true for shortening of the connecting alkanoyl chain between the guanidine moiety and the cyclohexyl ring (**12**→**11**→**10**→**9**).

Exchange of cyclohexyl against phenyl (**14**→**8**) reduced hH_2R potency without affecting efficacy. Shortening of the connecting chain (**8**→**7**) was also well tolerated. In the series of compounds with a phenyl ring and a connecting chain ranging from tetramethylene to none (**6**→**2**), minor changes in potency except for compound **2** and variable effects on efficacy were noted.

Overall, as is true for guanidines (Kelley et al., 2001) and AIPGs with two aromatic ring systems (Xie et al., 2006), AIPGs with a single ring system exhibited higher potencies and efficacies at gpH_2R-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$ (Table 1 and Fig. 3). However, among the series of aryl/diarylalkylguanidines ("guanidines"), AIPGs with two aromatic substituents and AIPGs with one substituent, the systematic difference between hH_2R and

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gpH_2R was the smallest (compare Tables 1 and 3 of this study with Fig. 6 and Table 2 in Kelley et al. (2001) and Fig. 2 and Table 1 in Xie et al. (2006)). Most notably, among compounds **2-14**, six derivatives (46% of the compound pool) (**3**, **4**, **6**, **9**, **11**, **12**) exhibited potencies that varied by just two-fold among hH_2R and gpH_2R, whereas for guanidines only one out of nine compounds (11% of the compound pool) (BU-E-43) fell in this group (Kelley et al., 2001). For AIPGs substituted with two aromatic ring systems just three out of twelve compounds (25% of the compound pool) (UR-PG137, UR-PG-55B and UR-PG153) were in this range (Xie et al., 2006).

Structure-activity relationships for the partial hH_1R agonism and gpH_1R antagonism of AIPGs 2-14 in the GTPase assay. In membranes expressing hH_1R (Fig. 2C) and gpH_1R (Fig. 2D), HIS activated GTP hydrolysis with an EC_{50} value of ~200 nM. At hH_1R UR-AK57 was a similarly potent agonist (EC_{50}, 280 nM) as HIS. The stimulatory effect of UR-AK57 on GTP hydrolysis catalyzed by gpH_1R was too small to assess potency. At hH_1R, UR-AK57 was a moderately strong partial agonist (E_{max}, 0.56), and at gpH_1R, UR-AK57 was only a very weak partial agonist (E_{max} 0.13). Among all AIPGs studied, compound **14** was the most potent and efficacious partial hH_1R agonist. Chain shortening (**9**, **10**, **13**), chain elongation (**12**) and methyl group removal (**11**) reduced agonist potency and efficacy. Exchange of the cyclohexyl ring (**9-14**) against a phenyl ring (**2-8**) reduced hH_1R agonism as well. Collectively, a 3-substituted butanoyl moiety connecting the guanidino group and the cyclohexyl ring are favorable for hH_1R agonism.

Most AIPGs, particularly compound **14**, exhibited much lower efficacies at gpH_1R than at hH_1R (Table 2). In fact, AIPGs were gpH_1R antagonists with affinities in the 0.5-2 μM range. UR-AK57 (**14**) exhibited ~3-fold higher affinity for hH_1R than for gpH_1R in the GTPase assay. Other AIPGs exhibited up to 15-fold higher affinity for gpH_1R than for hH_1R.

Inhibition of UR-AK57 (14)-stimulated GTP hydrolysis at hH_1R by H_1R antagonists. H_1R agonists of the 2-phenylistamine class are cationic-amphiphilic compounds and efficient direct G-protein activators in some systems (Seifert et al., 1994; Hagelüken et al., 1995). In order to exclude direct G-protein activation as mechanism for the GTPase stimulation by UR-AK57, which is cationic-amphiphilic as well, we studied the effects of the first-generation H_1R antagonists mepyramine, promethazine, diphenhydramine, triprolidine and cyproheptadine as well as the second-generation H_1R antagonists terfenadine and fexofenadine on GTP hydrolysis stimulated by UR-AK57 (1 μM) (Fig. 4). H_1R antagonists inhibited GTP hydrolysis in the order of potency promethazine > cyproheptadine > triprolidine > mepyramine > diphenhydramine > terfenadine >> fexofenadine. This order of potency fits exactly to the one observed for HIS-stimulated GTP hydrolysis at hH_1R (Seifert et al., 2003).

Affinities of HIS and AIPGs for hH_1R and gpH_1R in [^{3}H]mepyramine competition binding experiments. In the GTPase assay, we determined the agonist potencies of AIPGs at hH_1R, but for the gpH_1R, antagonist affinities had to be determined. Since agonist potencies depend on several factors including G-protein availability, those values cannot directly be compared with antagonist potencies (Seifert et al., 1999). Therefore, we compared potencies of representative AIPGs in the [^{3}H]mepyramine competition binding assay. All compounds inhibited [^{3}H]mepyramine binding according to monophasic isotherms (Table 3) that were insensitive to guanine nucleotides (data not shown). The latter findings indicate that ternary complex formation is not detected in this system, probably due to the low expression level of the insect G_q-protein (Houston et al., 2002). Among all compounds studied, UR-AK57 (**14**) exhibited the highest affinity for hH_1R. Chain shortening between the

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guanidino group and the cyclohexyl group (**10**, **13**) and substitution of the cyclohexyl ring by a phenyl ring (**5**, **6** and **8**) were unfavorable, whereas hH_1R tolerated chain elongation (**12**). At gpH_1R, UR-AK22 (**6**) exhibited the highest affinity within the compound pool, whereas UR-AK57 (**14**) ranged among the low-affinity compounds at this receptor. gpH_1R tolerated a phenyl ring (**5**, **6** and **8**), a methylene linker (**10**) and a trimethylene linker (**12**) better than hH_1R. In contrast, hH_1R tolerated the methyl-branched chain (**13** and **14**) better than gpH_1R.

Discussion

Historically, the availability of a generally applicable and reliable analysis system for the hH_2R was a substantial problem (Klinker et al., 1996; Dove et al., 2004). During the past years, our group has established fusion proteins of the hH_2R and $G_{s\alpha}$ as standard model for the analysis of both agonists and antagonists (Kelley et al., 2001; Wenzel-Seifert et al., 2001; Houston et al., 2002; Xie et al., 2006). UR-AK57 (**14**) is the most potent hH_2R agonist known so far, and among all bulky hH_2R agonists examined, it also exhibits one of the highest efficacies (Table 1) (Kelley et al., 2001; Xie et al., 2005). Thus, a 3-substituted butanoyl moiety connecting the cyclohexyl substituent and the guanidino group is optimal in affording high potency and efficacy at hH_2R. Probably, the cyclohexyl ring and butanoyl moiety of UR-AK57 form hydrophobic interactions with amino acid residues in tramsmembrane domains 3, 6 and 7, and Ala-271 in transmembrane domain 7 of hH_2R may be of particular importance in this respect (Kelley et al., 2001). It is noteworthy that hH_2R tolerated alterations of linker length between the acylguanidino group and the phenyl- or cylohexyl ring quite well (Table 1), indicative for conformational flexibility of hH_2R with this particular compound class. With diarylalkylguanidines as ligands, hH_2R exhibited lower overall conformational flexibility than gpH_2R (Kelley et al., 2001), but those guanidines are

also bulkier than the AIPGs studied herein. Thus, among the guanidines and AIPGs studied so far, UR-AK57 possesses the optimum properties in terms of hH_2R potency and efficacy.

The recently studied AIPGs with two aromatic ring substituents surpass UR-AK57 in terms of potency at gpH_2R-$G_{s\alpha S}$ (EC_{50} of UR-AK57 9 nM versus EC_{50} of UR-PG80, 6 nM). The opposite is true for hH_2R (EC_{50} of UR-AK57, 23 nM versus EC_{50} of UR-PG80, 78 nM). These data support the notion that hH_2R accommodates the 3-cyclohexylbutanoyl moiety of UR-AK57 particularly well. It is conceivable that Ala-271 is crucial in mediating the high-affinity interactions of phenyl- and cyclohexyl-substituted AIPGs with hH_2R, whereas gpH_2R bears an aspartate residue at this position, rendering hydrophobic interactions impossible (Kelley et al., 2001). However, in gpH_2R, alternative hydrophobic interactions of AIPGs with other as yet unidentified amino acids in transmembrane domains 3, 6 and 7 must take place since those compounds exhibit high affinity for gpH_2R as well.

Bulky guanidines are moderately potent H_1R antagonists, with arpromidine exhibiting a K_i value of 33 nM at gpH_1R in the [^{3}H]mepyramine competition binding assay (Seifert et al., 2003). At hH_1R, arpromidine is 10-fold less potent than at gpH_1R (K_i, 350 nM) (Seifert et al., 2003). Structural differences in transmembrane domain 2 play a crucial role for the differences in affinity of guanidines at the two H_1R receptor isoforms (Bruysters et al., 2005). The exchange of the guanidino group against an acylguanidino group decreases affinity for gpH_1R about 300-fold (K_i of UR-PG136 in the [^{3}H]mepyramine competition binding assay, 9.6 μM). Similar changes were observed for arpromidine versus UR-PG136 at hH_1R (Xie et al., 2006). These data show that AIPGs ensure excellent selectivity (> 100-fold) for H_2R isoforms relative to H_1R isoforms. However, while in terms of affinity for H_1R isoforms, AIPGs substituted with two aromatic ring systems were not particularly interesting, we noted that those compounds were weak partial hH_1R agonists with preference for gpH_1R in terms of agonist efficacy (Xie et al., 2006).

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Based on those observations we examined the effects of AIPGs substituted with a single aromatic/aliphatic substituent on H_1R isoforms. Unexpectedly, UR-AK57 turned out to be moderately strong partial hH_1R agonist exhibiting a potency that approaches that of HIS. In fact, in terms of efficacy (E_{max}, 0.56) and potency (EC_{50}, 280 nM) at hH_1R, UR-AK57 is comparable to the most potent derivatives of the 2-phenylhistamine class, which are classic H_1R agonists (Bakker et al., 2002; Pertz et al., 2004). Specifically, 2-(3-bromophenyl)histamine exhibits an efficacy of 0.73 and a potency of 210 nM at hH_1R (Seifert et al., 2003). In marked contrast, UR-AK57 is only a very weak partial agonist at gpH_1R with lower apparent affinity than for hH_1R in GTPase experiments (Table 2). Thus, UR-AK57 is the first synthetic H_1R agonist with higher potency and efficacy for hH_1R than gpH_1R.

Since AIPGs are cationic-amphiphilic and compounds with such properties can activate G-proteins directly (Seifert et al., 1994; Hagelüken et al., 1995), it was important to exclude the possibility of direct G-protein activation by AIPGs. Direct G-protein-stimulatory effects of histamine receptor ligands are usually observed at concentrations > 10-100 μM (Seifert et al., 1994; Hagelüken et al., 1995), but the stimulatory effects of UR-AK57 on GTP hydrolysis in membranes expressing hH_1R were already apparent at a concentration as low as 100 nM (Fig. 2C). The different concentration ranges argue against direct G-protein stimulation playing a part in the GTPase activation in hH_1R-expressing Sf9 membranes. The largely different effects of UR-AK57 on GTPase activity in Sf9 membranes expressing hH_1R and gpH_1R (compare Figs. 2C and 2D) also corroborate the notion that the stimulatory effects of the compound on hH_1R are not due to direct G-protein activation since Sf9 membranes harboring hH_1R and gpH_1R express the same type of endogenous G_q-protein (Houston et al., 2002). Finally, the studies with H_1R antagonists (Fig. 4) provided definitive proof that the

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pronounced stimulatory effect of UR-AK57 on GTPase activity is due to H_1R activation and no due to receptor-independent G-protein activation.

In the [^{3}H]mepyramine competition binding assay, UR-AK57 exhibited similar potency at hH_1R and gpH_1R (K_i, ~1 μM) (Table 3). The higher apparent affinity of the compound for hH_1R in the GTPase assay (EC_{50}, 280 nM) (Table 2) is probably due to the fact that in those studies, only the high-affinity (G-protein-coupled) UR-AK57-liganded hH_1R is assessed, whereas in the [^{3}H]mepyramine competition binding assay only the low-affinity (G-protein-uncoupled) UR-AK57-liganded hH_1R is assessed. This affinity difference between the two assays probably reflects the relative paucity of available G-proteins as coupling partners which are detected with greater sensitivity in the GTPase assay than in the binding assay. The similar affinity of UR-AK57 at gpH_1R in the GTPase- and [^{3}H]mepyramine competition binding assays compared to the different apparent affinities of this compound in the corresponding assays with hH_1R (Tables 2 and 3) further support the notion of a specific agonist action of UR-AK57 on hH_1R.

The structure-activity relationships of AIPGs for interaction with hH_1R and gpH_1R are different in terms of agonist efficacy and affinity in the GTPase and [^{3}H]mepyramine competition binding assay (Tables 2 and 3). Most importantly, a 3-substituted butanoyl moiety as is present in **14** is favorable for hH_1R agonism. These data indicate that it may become possible to synthesize bulky H_1R agonists with even greater preference for hH_1R relative to gpH_1R than UR-AK57.

Our present study demonstrates that the notion of bulky agonists exhibiting higher potencies and efficacies at gpH_1R and gpH_2R than at hH_1R and hH_2R, respectively (Kelley et al., 2001; Seifert et al., 2003; Xie et al., 2006), is actually not true. Specifically, several AIPGs substituted with a phenyl or cyclohexyl ring exhibit similar potencies at hH_2R and gpH_2R and include the most potent hH_2R agonist identified so far (Fig. 3 and Table 1). In

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terms of efficacy at hH_2R, UR-AK57 comes close to a full agonist as well. Most strikingly, UR-AK57 is also a potent and moderately strong partial hH_1R agonist with much higher efficacy than at gpH_1R (Figs. 2C and 2D). Thus, UR-AK57 constitutes an interesting starting point for the development of potent and efficacious hH_2R- and hH_1R agonists.

Not only may H_2R agonists be a good starting point for the development of H_1R agonists but conversely, H_1R agonists may also serve as template for the development of H_2R agonists. This notion is supported by the finding that N^{α}-(imidazolylethyl)histaprodifen, originally synthesized as H_1R agonist (Pertz et al., 2004), is a potent partial hH_1R agonist (EC_{50}, 0.24 μM; E_{max}, 0.84), and a potent partial hH_2R agonist (EC_{50}, 0.57 μM; E_{max}, 0.39) (Seifert et al., 2003). Our present data emphasize the importance of examining all potential ligands for the H_1R and H_2R both in the agonist- and antagonist mode for each receptor subtype and species isoform and not to extrapolate the putative ligand properties from previous studies obtained even with closely related compounds. We assume that the numerous compounds designed for agonistic activity at H_1R and H_2R (Bakker et al., 2002; Pertz et al., 2004; Dove et al., 2004), still hold many surprising pharmacological properties that have been missed so far because of incomplete analyses. In future studies we will systematically analyze agonist and antagonist effects of guanidines, AIPGs and histaprodifens at H_1R and H_2R species isoforms. In this analysis, we will include the human and guinea pig histamine receptor and the rat receptor as recent data point to unique pharmacological properties of the rat H_2R (Xie et al., 2006). In terms of future compound synthesis, pharmacophoric elements of the histaprodifens and 2-phenylhistamines will be combined with structural elements of **2-14**. Finally, the compounds analyzed in this paper will have to be examined in native systems.

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Footnotes

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Legends for Figures

Fig. 1. Structures of H_2R agonists. HIS (**1**) is the reference compound. Compounds **2-14** are AIPGs.

Fig. 2. Comparison of the agonistic effects of HIS and UR-AK57 (14) at H_1R- and H_2R species isoforms. Steady-state GTPase activity in Sf9 membranes expressing hH_2R-$G_{s\alpha S}$ (**A**), gpH_2R-$G_{s\alpha S}$ (**B**), hH_1R plus RGS protein 4 (**C**) or gpH_1R plus RGS protein 4 (**D**) was determined as described in *Methods*. Reaction mixtures contained HIS or UR-AK57 at the concentrations indicated on the abscissa to generate saturated concentration/response curves. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Data shown are the means ± SD of a representative experiment performed in triplicates. A summary of the results of 5-8 independent experiments is shown in Table 1.

Fig. 3. 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, 0.70 ± 0.14; $r^2 = 0.69$; $p = 0.0004$ (significant). **B**, correlation of potencies of AIPGs at hH_2R-$G_{s\alpha S}$ *versus* gpH_2R-$G_{s\alpha S}$. Slope, 0.96 ± 0.12; $r^2 = 0.86$; $p < 0.0001$ (significant).

Fig. 4. Inhibition of UR-AK57-stimulated GTP hydrolysis at hH_1R by H_1R antagonists.

Steady-state GTPase activity in Sf9 membranes expressing hH_1R plus RGS protein 4 was determined as described in *Methods*. Reaction mixtures contained UR-AK57 (1 μM) and various H_1R antagonists at the concentrations indicated at the abscissa. MEP, mepyramine; PRO, promethazine; TEF, terfenadine; FEX, fexofenadine; DPH, diphenhydramine; TRI, triprolidine; CYPH, cyproheptadine. Data were analyzed by non-linear regression and were best fit to sigmoid concentration/response curves. Data shown are the means ± SD of 3 independent experiments.

Table 1. Agonist potencies and efficacies of HIS 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**
2	**UR-AK41**	**0.50 ± 0.01***	**1,400 ± 37***	**86**	**0.84 ± 0.06**	**640 ± 170**	**190**	**2.19**
3	**UR-AK26**	**0.76 ± 0.01***	**150 ± 8***	**800**	**0.90 ± 0.01**	**76 ± 13**	**1,600**	**1.98**
4	**UR-AK51**	**0.84 ± 0.03***	**100 ± 16***	**1,200**	**1.05 ± 0.11**	**23 ± 1**	**5,200**	**4.35**
5	**UR-AK67**	**0.61 ± 0.01***	**67 ± 9***	**1,800**	**0.84 ± 0.01**	**21 ± 2**	**5,700**	**3.19**
6	**UR-AK22**	**0.60 ± 0.01***	**72 ± 23**	**1,700**	**0.80 ± 0.06**	**56 ± 5**	**2,100**	**1.29**
7	**UR-AK68**	**0.81 ± 0.03**	**75 ± 26***	**1,600**	**0.89 ± 0.03**	**29 ± 7**	**4,100**	**2.59**
8	**UR-AK24**	**0.87 ± 0.01***	**67 ± 2***	**1,800**	**1.03 ± 0.06**	**12 ± 1**	**10,000**	**5.58**
9	**UR-AK46**	**0.76 ± 0.05***	**38 ± 4***	**3,200**	**0.99 ± 0.08**	**21 ± 1**	**5,700**	**1.81**
10	**UR-AK62**	**0.87 ± 0.04**	**62 ± 6***	**1,900**	**1.01 ± 0.13**	**23 ± 1**	**5,200**	**2.70**
11	**UR-AK49**	**0.76 ± 0.04***	**46 ± 2***	**2,600**	**1.02 ± 0.11**	**22 ± 1**	**5,500**	**2.09**
12	**UR-AK64**	**0.66 ± 0.01***	**23 ± 2***	**5,200**	**0.84 ± 0.06**	**16 ± 2**	**7,500**	**1.44**
13	**UR-AK59**	**0.74 ± 0.06**	**99 ± 8***	**1,200**	**0.90 ± 0.08**	**37 ± 8**	**3,200**	**2.68**
14	**UR-AK57**	**0.87 ± 0.05**	**23 ± 3***	**5,200**	**1.11 ± 0.16**	**9 ± 1**	**13,300**	**2.56**

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 ~1.5 – 3.0 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-$G_{s\alpha S}$. Cpd., compound.

Table 2. Agonist potencies and efficacies of HIS and AIPGs and antagonist potencies of AIPGs at hH_1R and gpH_1R in the GTPase assay

		hH_1R		gpH_1R	
Cpd.		**efficacy**	**EC_{50} or K_B (nM)**	**efficacy**	**K_B (nM)**
1	HIS	1.00	190 ± 8.6	1.00	210 ± 5.3
3	UR-AK26	0.14 ± 0.05	n. d.	0.02 ± 0.01	n. d.
4	UR-AK51	0.21 ± 0.03	n. d.	0.01 ± 0.01	850 ± 80
5	UR-AK67	0.19 ± 0.01	n. d.	0.06 ± 0.03	720 ± 110
6	UR-AK22	0.15 ± 0.02	6,000 ± 200 (K_B)	0.06 ± 0.01	840 ± 170
7	UR-AK68	0.26 ± 0.01	3,500 ± 100	0.05 ± 0.02	2,000 ± 100
8	UR-AK24	0.35 ± 0.05	n. d.	0.01 ± 0.01	760 ± 70
9	UR-AK46	0.32 ± 0.11	13,000	0.23 ± 0.12	890 ± 90
10	UR-AK62	0.29 ± 0.02	n. d.	0.28 ± 0.04	720 ± 100
11	UR-AK49	0.24 ± 0.04	n. d.	0.11 ± 0.01	620 ± 50
12	UR-AK64	0.21 ± 0.04	n. d.	0.08 ± 0.02	530 ± 50
13	UR-AK59	0.32 ± 0.07	2,300 ± 360	0.21 ± 0.02	1,300 ± 190
14	UR-AK57	0.56 ± 0.06	280 ± 4	0.13 ± 0.04	750 ± 120

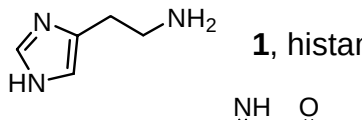
Steady-state GTPase activity in Sf9 membranes expressing hH_1R and gpH_1R in the presence of the RGS proteins 4 or 19 was determined as described in *Methods*. Reaction mixtures contained ligands at concentrations from 1 nM - 1 mM as appropriate to generate saturated concentration/response curves. Data 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.

Table 3. Affinities of HIS 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
5	UR-AK67	14 ± 3.7	14	2.2 ± 0.39	210	6.36
6	UR-AK22	2.9 ± 0.14	69	0.44 ± 0.14	1,000	6.59
8	UR-AK24	5.3 ± 0.20	38	2.6 ± 0.72	180	2.04
10	UR-AK62	7.0 ± 0.48	29	4.7 ± 0.63	98	1.48
12	UR-AK64	0.99 ± 0.22	200	0.35 ± 0.05	1,300	2.82
13	UR-AK59	6.0 ± 0.47	33	11 ± 2.6	42	0.56
14	UR-AK57	0.91 ± 0.16	220	1.6 ± 0.37	290	0.57

[^{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).

JPET #102897 Fig. 1



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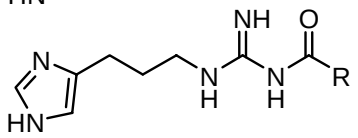
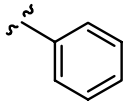
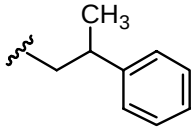
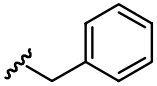
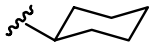
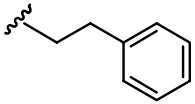
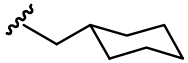
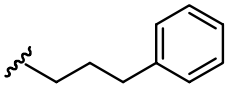
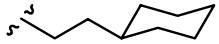
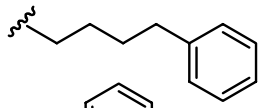
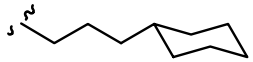
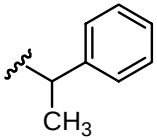
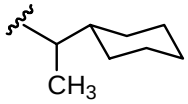
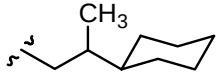
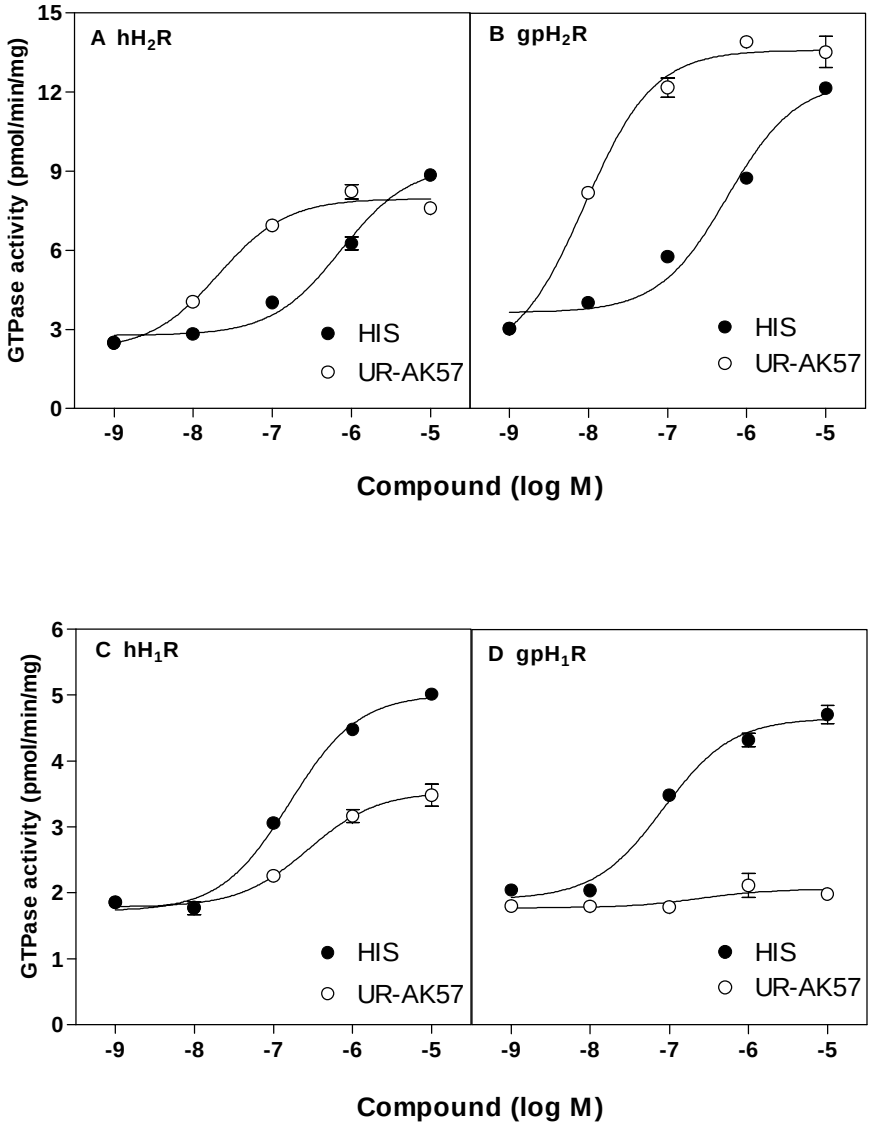


2-14

No.	R	No.	R
2, UR-AK41		**8**, UR-AK24	
3, UR-AK26		**9**, UR-AK46	
4, UR-AK51		**10**, UR-AK62	
5, UR-AK67		**11**, UR-AK49	
6, UR-AK22		**12**, UR-AK64	
7, UR-AK68		**13**, UR-AK59	
		14, UR-AK57	



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JPET #102897 **Fig. 3**

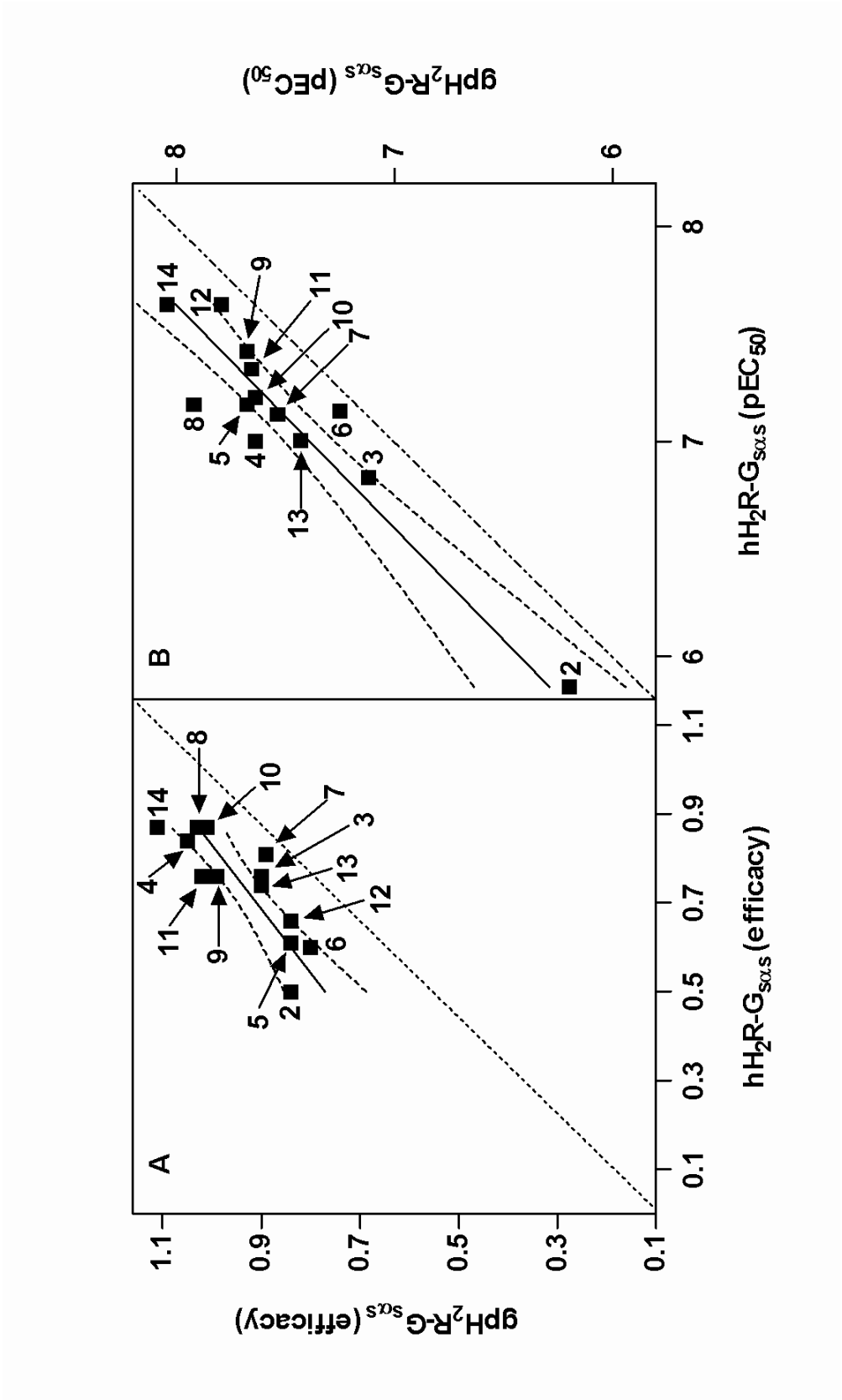

Fig. 4