Full Paper

Effects of Impromidine- and Arpromidine-Derived Guanidines on Recombinant Human and Guinea Pig Histamine H_1 and H_2 Receptors

Sheng-Xue Xie[1,2], Fabian Schalkhausser[3,4], Qi-Zhuang Ye[1,5], Roland Seifert[3,6], and Armin Buschauer[3]

[1] High Throughput Screening Laboratory, University of Kansas, Lawrence, USA
[2] Current address: Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, USA
[3] Institute of Pharmacy, University of Regensburg, Regensburg, Germany
[4] Current address: Siegfried Ltd., Zofingen, Switzerland
[5] Current address: Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, USA
[6] Department of Pharmacology and Toxicology, University of Kansas, Lawrence, USA

Imidazolylpropylguanidines derived from impromidine and arpromidine are more potent and efficacious agonists at the guinea pig histamine H_2 receptor (gpH_2R) than at the human H_2R (hH_2R) in the GTPase assay. Additionally, such guanidines are histamine H_1 receptor (H_1R) antagonists with preference for the human relative to the guinea pig receptor. The purpose of this study was to examine structure-activity relationships of guanidines at human and guinea pig H_1R and H_2R species isoforms expressed in Sf9 insect cells. Three impromidine analogues and six arpromidine analogues exhibited agonistic activity at H_2R and antagonistic activity at H_1R as assessed in the steady-state GTPase assay. Species selectivity of derivatives was similar as compared with the parent compounds. None of the structural modifications examined (different aromatic ring systems and different ring substituents) was superior in terms of H_2R potency and efficacy relative to impromidine and arpromidine, respectively. These data point to substantial structural constraints at the agonist binding site of H_2R. Guanidines exhibited distinct structure-activity relationships for H_1R antagonism in a radioligand competition binding assay and the GTPase assay and for H_1R inverse agonism. Our data indicate that it is difficult to obtain guanidine-type agonists with high potency and high efficacy for hH_2R, but those compounds may be useful tools for exploring the antagonist binding site and constitutive activity of H_1R.

Keywords: Arpromidine / Guanidines / Histamine H_1 receptor / Histamine H_2 receptor / Impromidine

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Introduction

Histamine (HA, **1**) exerts its biological effects through four receptor subtypes, designated as H_1, H_2, H_3, and H_4 receptors (H_1R, H_2R, H_3R, H_4R), respectively [1 – 3]. The H_1R couples to G_q-proteins, the H_2R couples to G_s-proteins, and the H_3R and H_4R couple to G_i/G_o-proteins. We are particularly interested in the development of H_2R agonists that could be used for the treatment of acute heart failure, acute promyelocytic leukemia, and inflammatory diseases [4, 5]. H_2R agonists are divided into two classes. The first class comprises small molecules related to HA **1** (Fig. 1) such as amthamine and dimaprit. The amino group of HA forms an ionic interaction with Asp-98 in transmembrane domain 3, and the imidazole ring interacts with Tyr-182 and Asp-186 in transmembrane domain

Correspondence: Prof. Dr. Armin Buschauer, Institute of Pharmacy, University of Regensburg, D-93040 Regensburg, Germany
E-mail: armin.buschauer@chemie.uni-regensburg.de
Fax: +49 941 943-4820

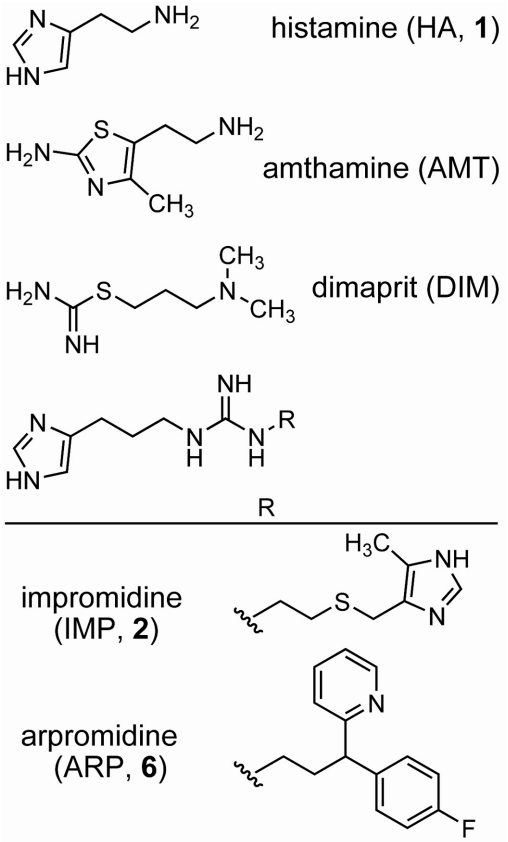
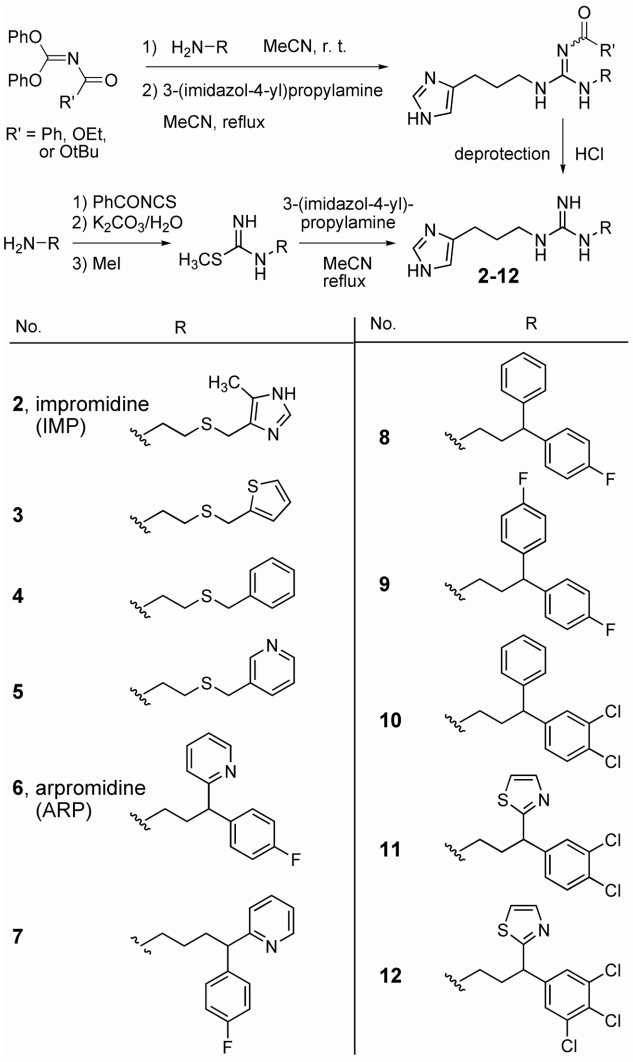


Figure 1. Structures of histamine **1**, amthamine, dimaprit, and the highly potent guanidine-type H_2R agonists impromidine **2** and arpromidine **6**.



Scheme 1. Synthetic pathways to the investigated guanidine-type histamine H_2 receptor agonists **2–12.**

5 [6, 7]. The second class of H_2R agonists consists of long-chained and more bulky molecules, impromidine (IMP, **2**) and arpromidine (ARP, **6**) (Fig. 1) being the prototypes [4, 8]. Highest potency is found for compounds with a three-membered instead of a two-membered carbon chain as in histamine connecting the imidazole ring and the basic group. The corresponding partial structures of impromidine and histamine are considered as functionally equivalent groups that are important for the receptor activation i.e., the guanidino group and the imidazolylpropyl moieties of IMP and ARP are supposed to form similar interactions with the H_2R as the amino group and imidazole groups of HA, respectively [5, 9]. Additionally, the 2-(5-methylimidazol-4-ylmethylthio)ethyl moiety of IMP and the 3-(4-fluorophenyl)-3-(2-pyridyl)propyl substituent of ARP interact with a pocket formed by multiple residues in transmembrane domains 3, 6, and 7 [9]. Traditionally, measurement of positive chronotropic effects in the guinea pig right atrium has been used as a read-out for the measurement of H_2R agonist potency and efficacy [1, 2, 4]. However, recent studies have revealed substantial pharmacological differences between hH_2R and gpH_2R recombinantly expressed in Sf9 insect cells [9, 10]. Specifically, at gpH_2R, IMP and ARP are full agonists and up to 30-fold more potent than HA. In contrast, at hH_2R, IMP and ARP are only partial agonists and just sixfold more potent than HA [9, 10]. Modeling and mutagenesis studies showed that the pharmacological differences between hH_2R and gpH_2R are due to the non-conserved Asp-271 in transmembrane domain 7 of gpH_2R (Ala-271 in hH_2R) and Tyr-17 in transmembrane domain 1 of gpH_2R (Cys-17 in hH_2R). In addition, IMP and ARP-derived compounds are H_1R antagonists with preference for gpH_1R relative to hH_1R, Asn-84 in transmembrane domain 2 playing a crucial role in conferring species-selectivity to H_1R ligands [11, 12].

Table 1. Agonist potencies and efficacies of HA and guanidines 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-$G_{s\alpha S}$
Compd.	**Efficacy**	EC_{50} (nM)	**Rel. pot.**	**Efficacy**	**EC_{50} (nM)**	**Rel. pot.**	
1	1.00	1,200 ± 300	100	1.00	1,200 ± 200	100	1.00
2	0.82 ± 0.04[a)]	210 ± 20[a)]	570	0.99 ± 0.09	42 ± 10	2,900	5.00
3	0.74 ± 0.06[a)]	230 ± 20[a)]	520	1.00 ± 0.01	51 ± 12	2,400	4.51
4	0.77 ± 0.14[a)]	420 ± 57[a)]	290	0.97 ± 0.01	120 ± 16	1,000	3.50
5	0.83 ± 0.05[a)]	510 ± 23[a)]	240	0.92 ± 0.03	170 ± 15	710	2.96
6	0.80 ± 0.05[a)]	180 ± 50[a)]	670	1.00 ± 0.06	65 ± 8	1,800	2.77
7	0.58 ± 0.02[a)]	600 ± 25[a)]	200	0.85 ± 0.09	170 ± 23	710	3.53
8	0.90 ± 0.13	470 ± 83[a)]	260	1.03 ± 0.08	120 ± 27	1,000	4.01
9	0.84 ± 0.05[a)]	360 ± 15[a)]	330	1.03 ± 0.10	83 ± 1	1,500	4.30
10	0.71 ± 0.02[a)]	710 ± 100[a)]	170	0.98 ± 0.01	240 ± 6	500	2.95
11	0.51 ± 0.14[a)]	810 ± 130[a)]	150	0.87 ± 0.09	210 ± 9	570	3.86
12	0.50 ± 0.11[a)]	790 ± 110[a)]	150	0.84 ± 0.04	200 ± 15	600	3.95

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 Experimental (section 3). Reaction mixtures contained ligands at concentrations from 1 nM to 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.

[a)] $p < 0.05$ 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.

Our long-term goal is to obtain highly potent and efficacious hH_2R agonists. The purpose of this study was to extend the structure-activity relationships for IMP-derived guanidines **2–5** and ARP-derived guanidines **6–12** (Scheme 1) for agonistic activity at hH_2R and gpH_2R and antagonistic activity at hH_1R and gpH_1R. In case of H_2R, fusion proteins of the receptor and short splice variant of $G_{s\alpha}$ protein (hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$) were used to determine agonist-stimulated high-affinity GTP hydrolysis with high sensitivity [9,10]. In case of H_1R, we measured GTPase activity of the receptor coupled to insect cell G_q-proteins, enhancing the signal with regulator of G-protein signaling (RGS) proteins [11, 13].

Results and discussion

Chemistry

The guanidine-type histamine H_2R agonists were accessible according to the synthetic pathways outlined in Scheme 1 following the procedures described for **2–10** [14–17]. The guanidines **11** and **12** [18] were synthesized via the corresponding Boc-protected guanidines by stepwise aminolysis of *tert*-butyl diphenoxymethylidenecarbamate as reported for structurally related alkyl guanidine-*N*-carboxylates [19], followed by deprotection with hydrochloric acid. The pertinent phenyl(thiazol-2-yl)propylamines were prepared from the corresponding phenyl(thiazol-2-yl)ketones in analogy to a previously described method [20].

Pharmacology

Analysis of the interaction of histamine and guanidines 2-12 with hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$

HA activated the GTPase activity of hH_2R-$G_{s\alpha S}$ and gpH_2R-$G_{s\alpha S}$ with similar potency and was a full agonist (Table 1). IMP **2** activated hH_2R-$G_{s\alpha S}$ with a ~ sixfold higher potency than HA and was a strong partial agonist. Substitution of the methylimidazolyl moiety of IMP **2** by thiophene **3** slightly reduced efficacy but not potency. The introduction of a phenyl ring **4** reduced the potency and efficacy, whereas a pyridyl ring **5** reduced only potency. At gpH_2R-$G_{s\alpha S}$, IMP and its derivatives **2–5** were all more potent and efficacious than at hH_2R-$G_{s\alpha S}$. The various ring substitutions had no effect on efficacy but similar to hH_2R-$G_{s\alpha S}$, introduction of a phenyl ring **4** or pyridyl ring **5** reduced potency.

At hH_2R-$G_{s\alpha S}$, ARP, bearing a 4-fluorophenyl group **6**, was similarly potent and efficacious as IMP **2**. Extension of the chain length in **6**, resulting in the higher homologue **7**, reduced both potency and efficacy. Substitution of the pyridyl ring against phenyl **8** slightly increased efficacy but reduced potency. Substitution of the additional phenyl ring with 4-fluoro (**8** → **9**) slightly reduced the effi-

cacy with a small effect on potency. Substitution of the 4-fluorophenyl group of ARP **6** with 3,4-dichlorophenyl **10** had a negative impact in terms of potency and efficacy. Among ARP derivatives substituted with a thiazole ring **11**, **12**, 3,4,5-trichloro- and 4,5-dichloro substitutions were unfavorable in terms of agonist potency and efficacy.

At gpH_2R-$G_{s\alpha S}$, ARP **6** was slightly less potent than IMP **2** but similarly efficacious. Potencies and efficacies of ARP-derived guanidines **7**–**12** were higher at gpH_2R-$G_{s\alpha S}$ than at hH_2R-$G_{s\alpha S}$. The substitutions in **7**, **11**, and **12** that exhibited a negative impact on efficacy at hH_2R-$G_{s\alpha S}$ also had a negative impact on efficacy at gpH_2R-$G_{s\alpha S}$. Similarly to the observations made for hH_2R-$G_{s\alpha S}$, ARP-derived guanidines **7**–**12** were less potent than the parent compound **6**.

Analysis of the effects of histamine and guanidines at hH_1R and gpH_1R

The interaction of ligands with H_1R was examined in a radioligand competition assay, using the antagonist [^{3}H]mepyramine as probe. Guanidine **6** (ARP) exhibited the highest affinity for hH_1R among all compounds examined (Table 2). The affinity of ARP for hH_1R surpassed the affinity of HA by almost sixfold. Among ARP derivatives, extension of the connecting chain in **6** by one methylene group **7** had the most pronounced negative impact on affinity. IMP and its analogue **3** showed up to fivefold lower affinity for hH_1R than HA.

The affinities of all ligands studied were significantly different at hH_1R and gpH_1R. HA exhibited a lower affinity for gpH_1R than for hH_1R, whereas the opposite was true for guanidines **2**, **3**, **6**, **7**, **9**, and **10**. Among the compounds studied, ARP showed the highest affinity for gpH_1R, being 150-fold more potent than HA. All substitutions examined in guanidines (**2** *versus* **3** and **6** *versus* **7**, **9**, and **10**) reduced affinity for gpH_1R. Like ARP **6**, guanidine **9** exhibited substantial selectivity for gpH_1R relative to hH_1R, whereas species-selectivity for **3** and **7** was just twofold.

In a recent study, we showed that N^G-acylated imidazolylpropylguanidines are potent hH_2R- and gpH_2R agonists and partial hH_1R agonists [10]. These data prompted us to address the question whether IMP- and ARP-derived guanidines exhibit agonistic effects at hH_1R as well. However, agonistic activity of guanidines at hH_1R was only minimal for compound **3** and virtually absent for **4** and **11** (Table 3). Intriguingly, for **6**, **7**, and **9**, inverse agonistic activity was detected as reflected by a decrease of basal GTPase activity. At gpH_1R, **3** lacked agonistic activity, but similarly to the data obtained with hH_1R, **6**, **7**, and **9** showed inverse agonistic activity at gpH_1R. Compound **9** reduced basal GTP hydrolysis in membranes expressing gpH_1R with an IC_{50} value of 84 ± 12 nM (n = 3). This value is consistent with the K_B value determined in functional competition experiments of **9** with HA (see below). Moreover, the neutral antagonist **3** (10 μM) shifted the IC_{50} of **9** to 1.5 ± 0.2 μM (n = 3). Collectively, our data corroborate the notion that hH_1R exhibits constitutive, i.e. agonist-independent, activity and that several guanidines are inverse H_1R agonists [11].

Table 2. Affinities of HA and guanidines at hH_1R and gpH_1R in the [^{3}H]mepyramine competition binding assay.

Compd.	hH_1R		gpH_1R		K_i hH_1R/ K_i gpH_1R
	K_i (μM)	Rel. affinity	K_i (μM)	Rel. affinity	
1 (HA)	2.0 ± 0.19[a)]	100	4.6 ± 0.24	100	0.43
2	6.0 ± 1.3[a)]	33	0.92 ± 0.14	500	6.53
3	9.1 ± 0.4[a)]	22	4.0 ± 1.1	120	2.23
6	0.34 ± 0.08[a)]	590	0.03 ± 0.01	15,000	11.3
7	2.6 ± 0.1[a)]	77	1.2 ± 0.1	380	2.17
9	1.2 ± 0.1[a)]	170	0.11 ± 0.01	4,200	10.9
10	6.6 ± 0.2[a)]	330	1.5 ± 0.03	310	4.40

[^{3}H]Mepyramine competition binding in Sf9 membranes expressing hH_1R or gpH_1R with RGS4 or RGS19 was determined as described in Experimental (section 3). Reaction mixtures contained Sf9 membranes (20–25 μg of protein), 2 nM [^{3}H]mepyramine and unlabeled guanidines at concentrations of 10 nM to 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 (rel. affinity) of HA was set 100, and the affinities of other ligands were referred to this value.

[a)] $p < 0.05$ for comparison of hH_1R and gpH_1R.

Finally, we also examined the antagonistic effects of guanidines in a functional assay, determining inhibition of HA-stimulated high-affinity GTP hydrolysis (Table 3). Similar to the data obtained in the radioligand competition assay, ARP **6** exhibited the highest affinity for hH_1R among all guanidines studied. Except for the higher homologue **7**, ARP derivatives exhibited lower antagonistic potency at hH_1R than the parent compound. The IMP derivatives **3** and **4** showed low antagonistic affinity for hH_1R as well. With respect to gpH_1R, ARP **6** was the most potent antagonist as well. The structural variations in **7** and **9** had little impact on antagonist potency, but substitution of the phenyl ring with chlorine **10**, **11** had a negative impact on antagonist affinity. IMP derivatives **3** and **4** exhibited about 10-fold lower antagonistic potency than ARP at gpH_1R.

H_2R agonists are interesting potential drugs for the treatment of various human disorders including acute heart failure, acute promyelocytic leukemia and inflam-

Table 3. Antagonist potencies and agonist/inverse agonist efficacies of guanidines at hH_1R and gpH_1R in the GTPase assay

Compd.		hH_1R		gpH_1R		K_B hH_1R/ K_B gpH_1R
		K_B (nM)	Efficacy	K_B (nM)	Efficacy	
3	BU-E-23	4,000 ± 500[a)]	0.07 ± 0.04	400 ± 180	0.00 ± 0.03	10.0
4	BU-E-14	2,600 ± 210[a)]	0.04 ± 0.02	560 ± 180	0.04 ± 0.03	4.64
6	ARP	320 ± 80[a)]	−0.11 ± 0.05	49 ± 13	−0.12 ± 0.06	6.53
7	BU-E-84	370 ± 120[a)]	−0.16 ± 0.07	53 ± 4	−0.18 ± 0.04	6.98
9	FrA 19	660 ± 48[a)]	−0.15 ± 0.02	72 ± 3	−0.17 ± 0.06	9.12
10	BU-E-82	1,300 ± 310[a)]	−0.04 ± 0.02	890 ± 90	−0.04 ± 0.03	1.46
11	D287	1,200 ± 310[a)]	0.02 ± 0.01	410 ± 120	0.03 ± 0.02	2.93

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 Experimental (section 3). Reaction mixtures contained HA (1 μM) and guanidines at concentrations from 1 nM to 100 μM as appropriate to generate saturated inhibition 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 set 1.00. The E_{max} values of other compounds (examined at a fixed concentration of 10 μM) were referred to this value. Data shown are the means ± SD of 5–8 experiments performed in duplicates each.

[a)] $p < 0.05$ for comparison of hH_1R and gpH_1R.

matory diseases [4, 5]. In order to come closer to these ambitious goals, it is necessary to develop highly potent and efficacious hH_2R agonists. While initial studies with the guinea pig atrium showed that the guanidines IMP **2** and particularly ARP **6** are highly potent H_2R agonists [4, 8], subsequent studies with human cell systems were rather disappointing since IMP and ARP showed reduced efficacy and potency compared to the guinea pig atrium [21, 22]. The unfavorable pharmacological properties of the available H_2R agonists in human systems substantially delayed the further development of agonists, particularly because the molecular basis for the apparent pharmacological difference between hH_2R and gpH_2R remained elusive. Finally, with a lag period of almost a decade, it became clear that two defined amino acid differences in transmembrane domains 1 and 7 between hH_2R and gpH_2R account for the pharmacological differences [9]. These advances rekindled interest in the development of H_2R agonists [5, 10].

ARP, bearing a 4-fluorophenyl group at the guanidino group, is one of the most potent hH_2R agonists known so far, but it is still only a partial agonist and less potent than at gpH_2R (Table 1) [9]. Since substitution of 4-fluorophenyl by 4-chloro and 4-bromo slightly enhanced potency [9], we explored several halogen ring substitutions in our present study. Unfortunately, various modifications including introduction of a second 4-fluorophenyl group **9** and introduction of a 3,4-dichloro- or a 3,4,5-trichlorophenyl group **10–12** had negative rather than positive effects on agonist potency and efficacy at hH_2R. However, this result was not completely unexpected since a 3,4-difluoro substitution also slightly reduced agonist potency at hH_2R [9]. Other modifications such as substitution of pyridyl by phenyl **6**, **8** increased efficacy, but only at the expense of potency. The newly introduced modifications **8–12** in ARP did not enhance potency and efficacy at gpH_2R either. At gpH_2R, ARP derivatives studied were more potent and efficacious than at hH_2R, pointing to a systematic difference in interaction of agonists with the receptor in the two species. Only with respect to efficacy of **8**, there was no statistically significant difference between hH_2R and gpH_2R and no compound showed preference for hH_2R relative to gpH_2R.

The situation regarding structural modifications using IMP as starting point was similar to what we observed for the ARP series. Specifically ring substitutions in IMP (thiophene, phenyl, and pyridyl) **3–5** were not advantageous with respect to potency at hH_2R and gpH_2R. Additionally, the ring substitutions did not increase but rather tended to decrease agonist efficacy at hH_2R and gpH_2R. Although, on first glance, these results may seem disappointing, the data are actually very helpful for future ligand design. Specifically, our results clearly point to substantial structural constraints in the agonist-binding site both in hH_2R and gpH_2R. As a consequence of our data, it is probably not a promising strategy to further introduce gradual structural changes in the aromatic ring substituents of guanidines in order to obtain highly potent and efficacious hH_2R agonists. Rather, future studies should examine more drastic structural changes such as the introduction of saturated ring systems. Based on the present data, it is evident that the

achievement of the long-term goal, i.e. the availability of highly potent and efficacious hH_2R agonists, is difficult. Probably, the most efficient strategy will be to perform parallel molecular modeling, compound synthesis, and site-directed mutagenesis studies to fully understand the molecular mechanisms of agonist / H_2R interactions. The detailed analysis of other H_2R species isoforms including those of rat and dog [1, 2], will be very informative in this respect, too. Moreover, there are a number of amino acid differences between hH_2R and gpH_2R in the *N*-terminus, the second intracellular loop, and the *C*-terminus which could contribute to differences in agonist binding and/or G-protein coupling [9]. Understanding the functional relevance of these structural differences between receptor isoforms will be important for future agonist synthesis as well.

ARP-derived guanidines but not IMP-derived guanidines are also moderately potent H_1R antagonists (Tables 2 and 3). These data show that H_1R readily accommodates the second aromatic ring system present in ARP derivatives and that interaction of the aromatic rings with H_1R contributes substantially to antagonist-affinity. In terms of potential therapeutic application of ARP derivatives for inflammatory diseases, H_1R antagonism is actually an interesting property since H_2R agonism and H_1R antagonism should result in at least additive anti-inflammatory effects [1, 2, 21]. The gpH_2R shows a three- to fivefold higher affinity for guanidines than hH_2R (Table 1), but for H_1R species isoforms, the affinity difference varies from 1.5- to 10-fold (Tables 2 and 3). These data show that the specific aromatic ring systems and their substituents have a much greater impact on H_1R- than H_2R-affinity. Most notably, **10**, bearing a phenyl- and a 3,4-dichlorophenyl group, exhibits very similar affinity at hH_1R and gpH_1R, whereas **9**, bearing two 4-fluorophenyl groups, exhibits 10-fold preference for gpH_1R compared to hH_1R. Thus, ARP-derived guanidines may become very valuable tools to explore the antagonist binding site of H_1R.

In this context, it should be noted that the antagonist affinity ratios for the hH_1R species isoforms in the [^{3}H]mepyramine competition binding assay and the GTPase inhibition assay show some differences (Tables 2 and 3). For example, in the competition binding assay, **8** exhibits similar affinity at both receptor isoforms, whereas in the GTPase inhibition assay, the affinity-difference is more than fourfold. A possible explanation for these differences is that the two assays assess different H_1R populations with different ligand affinities, i.e. the [^{3}H]mepyramine-bound H_1R (competition binding assays) and the HA-bound H_1R (GTPase inhibition assay). Thus, guanidines may also become valuable tools to explore multiple ligand-specific H_1R conformations. The usefulness of guanidines for the exploration of ligand-specific H_2R conformations was already established in a previous study [9].

A last aspect that needs to be discussed concerns the constitutive, i.e. agonist-independent, activity of the H_1R. It is known that several H_1R antagonists act as inverse agonists as is reflected by a decrease in basal G-protein activity [11, 23]. However, to this end, specific structure-activity relationships for inverse agonistic activity have remained largely unexplored. Our present study shows that IMP derivatives **3, 4** lack inverse agonistic H_1R activity, whereas certain ARP derivatives **6, 7, 9** clearly display inverse agonism (Table 3). These data indicate that two aromatic ring systems at the guanidino group are required for inverse agonism. However, ARP derivatives **10** and **11** lacked inverse agonistic activity. An important difference between the latter two ARP derivatives and the former ARP derivatives is that **6**, **7**, and **9** bear a fluorophenyl substituent, whereas **10** and **11** bear chlorophenyl substituents. Thus, ARP-derived guanidines will also help us elucidate the structural requirement for inverse agonism at H_1R and explore the possible physiological function of H_1R constitutive activity.

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Experimental

Chemistry

Melting points (uncorrected) were determined with a Büchi 530 apparatus (Büchi, Flawil, Switzerland). ^{1}H-NMR (250 MHz) were recorded on a Bruker WM 250 NMR spectrometer (Bruker, Karlsruhe, Germany) (chemical shift δ in ppm, relative to tetramethylsilane, J in Hz, s = singulett, d = doublet, t = triplett, m = multiplet, br = broad). Elemental analyses (C, H, N) were performed by the Microanalytical Laboratory of the University of Regensburg. $^+$FAB mass spectra (methanol, glycerol, xenon) were recorded using a Finnigan MAT 95 mass spectrometer (Bremen, Germany). Thin-layer chromatography (TLC) was done on silica gel 60 F_{254} (Merck, Darmstadt, Germany) coated on aluminium sheets. Chromatographic separations on a preparative scale were performed with a Chromatotron, model 8924 (Harrison Research, Palo Alto, CA, USA) on 2 or 4 mm layers of silica gel 60 PF_{254} containing gypsum (Merck). The analytical HPLC system consisted of a 655A-12 Liquid Chromatograph (Merck), a L-5000 LC controller

(Merck), a L-4250 UV-VIS detector (Merck), a 655A-40 auto sampler (Merck), a D2000 Chromo-Integrator (Merck), a LiChrosorb RP18 (Merck) 7 µm column (250 mm × 4 mm) using mixtures of MeOH and 0.1% aqueous trifluoroacetic acid (TFA) as eluent, flow rate: 1 mL/min.

The guanidines **2 – 10** were synthesized as described elsewhere [4, 14 – 17], the compounds **11, 12** were prepared by analogy with known procedures as outlined below. The purity of the pharmacologically investigated compounds was ≥ 98% unless otherwise indicated, as determined by high-performance liquid chromatography or capillary electrophoresis according to previously described methods [24, 25].

*N^1-[3-(3,4-Dichlorophenyl)-3-(2-thiazolyl)propyl]-N^2-[3-(1H-imidazol-4-yl)propyl]guanidine **11** and N^1-[3-(3,4,5-trichlorophenyl)-3-(2-thiazolyl)propyl]-N^2-[3-(1H-imidazol-4-yl)propyl]guanidine **12***

3-(3,4-Dichlorophenyl)-3-(2-thiazolyl)propylamine and 3-(3,4,5-trichlorophenyl)-3-(2-thiazolyl)propylamine were prepared from the corresponding aromatic ketones via condensation with diethyl cyanomethanephosponate and stepwise reduction of the double bond and the nitrile group; see procedure described in [19]. The pertinent amine (2 mmol) and diphenoxymethylenecarbamic acid *t*-butyl ester (626 mg, 2 mmol) were stirred in acetonitrile (10 mL) for 10 min. After addition of 3-(1*H*-imidazol-4-yl)propylamine (250 mg, 2 mmol) the mixture was heated to reflux for 3 – 5 h (control by TLC). The solution was evaporated *in vacuo*, the residue was taken up in 20 mL of 5% acetic acid, phenol was removed by extraction with diethyl ether, and after basification with aqueous ammonia the intermediate, the corresponding guanidine-*N*-carboxylic acid *t*-butyl ester, was extracted with methylene chloride and isolated chromatographically from the dried (Na_2SO_4) and evaporated organic layer (Chromatotron, eluent: $CHCl_3$ then $CHCl_3$/MeOH 1 : 1, NH_3 atmosphere).

1,1-Dimethylethyl {[3-(3,4-dichlorophenyl)-3-(2-thiazolyl)propylamino][3-(1H-imidazol-4-yl)propylamino]methylidene}carbamate

Yield 63%, m. p. 98°C (product stirred with MeOH/hexane); $^+$FAB-MS: m/z (% rel. intensity) = 537 [MH^+] (60), 437 [MH – Boc]$^+$, (100). Analysis $C_{24}H_{30}Cl_2N_6O_2S \times 0.5\ CH_3OH$ (553.5). Anal. calcd.: C 53.16, H 5.83, N 15.18; found: C 53.06, H 5.86, N 14.96. HPLC: retention time (t_R) 28.34 min, eluting with MeOH/0.1% aqueous TFA, 50 : 50.

1,1-Dimethylethyl {[3-(3,4,5-trichlorophenyl)-3-(2-thiazolyl)propylamino][3-(1H-imidazol-4-yl)propylamino]methylidene}carbamate

Yield 44%, m. p. 78°C; $^+$FAB-MS: m/z (% rel. intensity) = 571 [MH^+] (68), 471 [MH – Boc]$^+$ (100), analysis $C_{24}H_{29}Cl_3N_6O_2S \times CH_3OH$ (604.0). Anal. calcd.: C 49.71, H 5.51, N 13.91; found: C 49.66, H 5.17, N 13.69. HPLC: t_R 5.10 and 15.83 min eluting with MeOH/0.1% aqueous TFA, 70 : 30 and 60 : 40, respectively.

The Boc-protected intermediates were stirred with 5 mL of 1 M hydrochloric acid at 70°C for 1 h, evaporated to dryness to obtain the trihydrochlorides of **11** and **12** as hygroscopic solid **11** or dry foam **12**.

11: Yield 95%; m. p. 125°C; analysis, $C_{19}H_{22}Cl_2N_6S \times 3HCl \times 2H_2O$ (582.8). Anal. calcd.: C 39.16, H 5.02, N 14.42; found: C 38.72, H 5.37, N 14.13. HPLC: t_R 8.66 and 20.09 min eluting with MeOH / 0.1% aqueous TFA, 50 : 50 and 40 60, respectively. ^{1}H-NMR [D_2O]: δ (ppm) = 8.47 (1H, d, J = 1.5 Hz), 7.74 (1H, d, J = 3.7 Hz), 7.58 (1H, d, J = 3.6), 7.47 (1H, d, J = 2.2 Hz), 7.42 (1H, d, J = 8.3 Hz), 7.20 (1H, dd, J = 8.3 Hz, J = 2.2 Hz), 7.10 (1H, d, J = 1.0 Hz), 4.59 – 4.50 (1H, m), 3.25-3.13 (2H, m), 2.93 (2H, t, J = 7.2 Hz), 2.59 (2H, t, J = 7.5 Hz), 2.49-2.37 (2H, m), 1.71 (2H, tt, J = 7.5 Hz, J = 7.2 Hz). $^+$FAB-MS: m/z (% rel. intensity) = 437 [MH^+] (100).

12: Yield 93%, hygroscopic foam, $C_{19}H_{21}Cl_3N_6S \times 3HCl$ (581.2). Purity > 95%, determined by HPLC: t_R 16.57 min, eluent MeOH/0.1% aqueous TFA, 50 : 50. ^{1}H-NMR: δ (ppm) = 8.47 (1H, d, J = 1.39 Hz), 7.69 (1H, d, J = 3.5 Hz), 7.51 (1H, d, J = 3.5 Hz), 7.42 (2H, s), 7.10 (1H, d, J = 1.0 Hz), 4.53 – 4.43 (1H, m), 3.20 – 3.11 (2H, m), 2.92 (2H, t, J = 7.1 Hz), 2.59 (2H, t, J = 7.7 Hz), 2.49-2.33 (2H, m), 1.70 (2H, tt, J = 7.7 Hz, J = 7.1 Hz). $^+$FAB-MS: m/z (% rel. intensity) = 471 [MH^+] (100).

Pharmacology

Construction of baculoviruses encoding hH_2R-$G_{s\alpha S}$, gpH_2R-$G_{s\alpha S}$, hH_1R, and gpH_1R was described previously [9]. Baculoviruses encoding RGS proteins 4 and 19 were a gift from Dr. E. Ross (Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX, USA). Sources of other materials are described elsewhere [9, 13]. Baculovirus infection and culture of Sf9 cells and membrane preparation were performed as described [9]. 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 [9]. H_1R expression levels were 4 – 6 pmol/mg as assessed by [^{3}H]mepyramine saturation binding [11].

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 [9, 11]. 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 (BSA) 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°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.

Radioligand 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 [11]. 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, USA). All analyses of experimental data were performed with the Prism 4.02 software

(GraphPad-Prism, San Diego, CA, USA). K_i and K_B values were calculated using the Cheng and Prusoff equation [26]. Statistical comparisons were performed with the *t*-test.

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